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(54) **METHOD OF PREPARING IMMUNO-REGULATORY DENDRITIC CELLS AND THE USE THEREOF**

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(52) **U.S. Cl.** **435/372**

(57) **ABSTRACT**

This invention provides: a therapeutic agent for graft rejection, graft-versus-host disease, autoimmune disease, allergic disease, or other diseases comprising dendritic cells (DCs) induced under culture conditions comprising both IL-10 and TGF-β or DCs prepared by adding inflammatory stimulation (e.g., TNF-α or LPS) to the aforementioned DCs and, if necessary, an antigen associated with a target disease; a method of inducing human immunoregulatory dendritic cells by culturing human dendritic cells or their precursor cells in vitro with cytokines comprising at least IL-10 and TGF-β; human immunoregulatory dendritic cells obtained by such method; and a pharmaceutical composition comprising such human immunoregulatory dendritic cells.

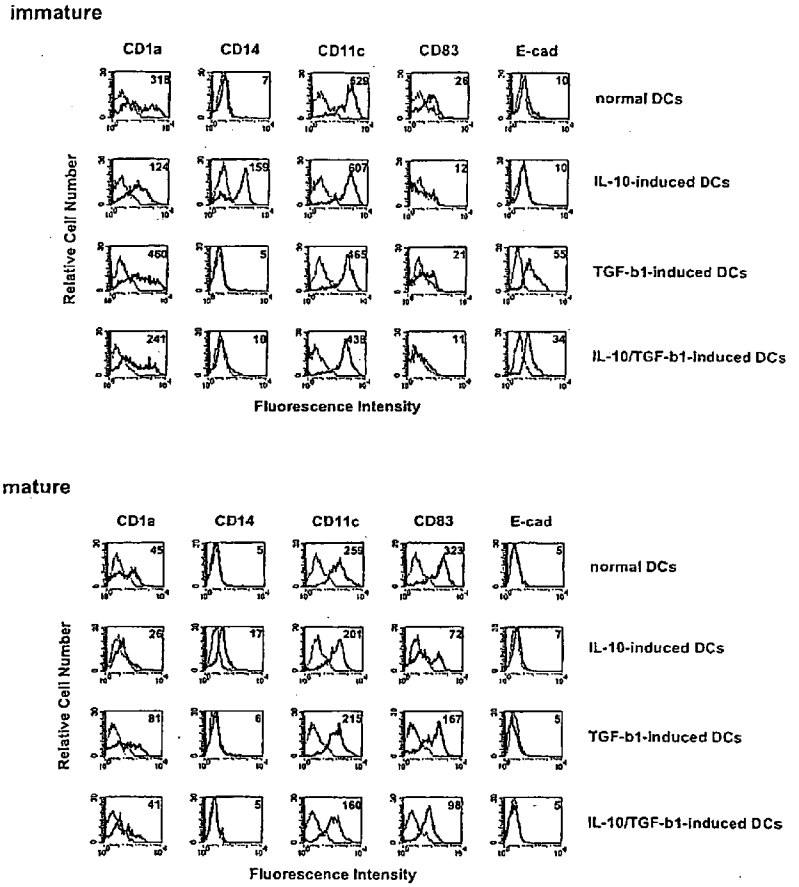


Fig. 1A

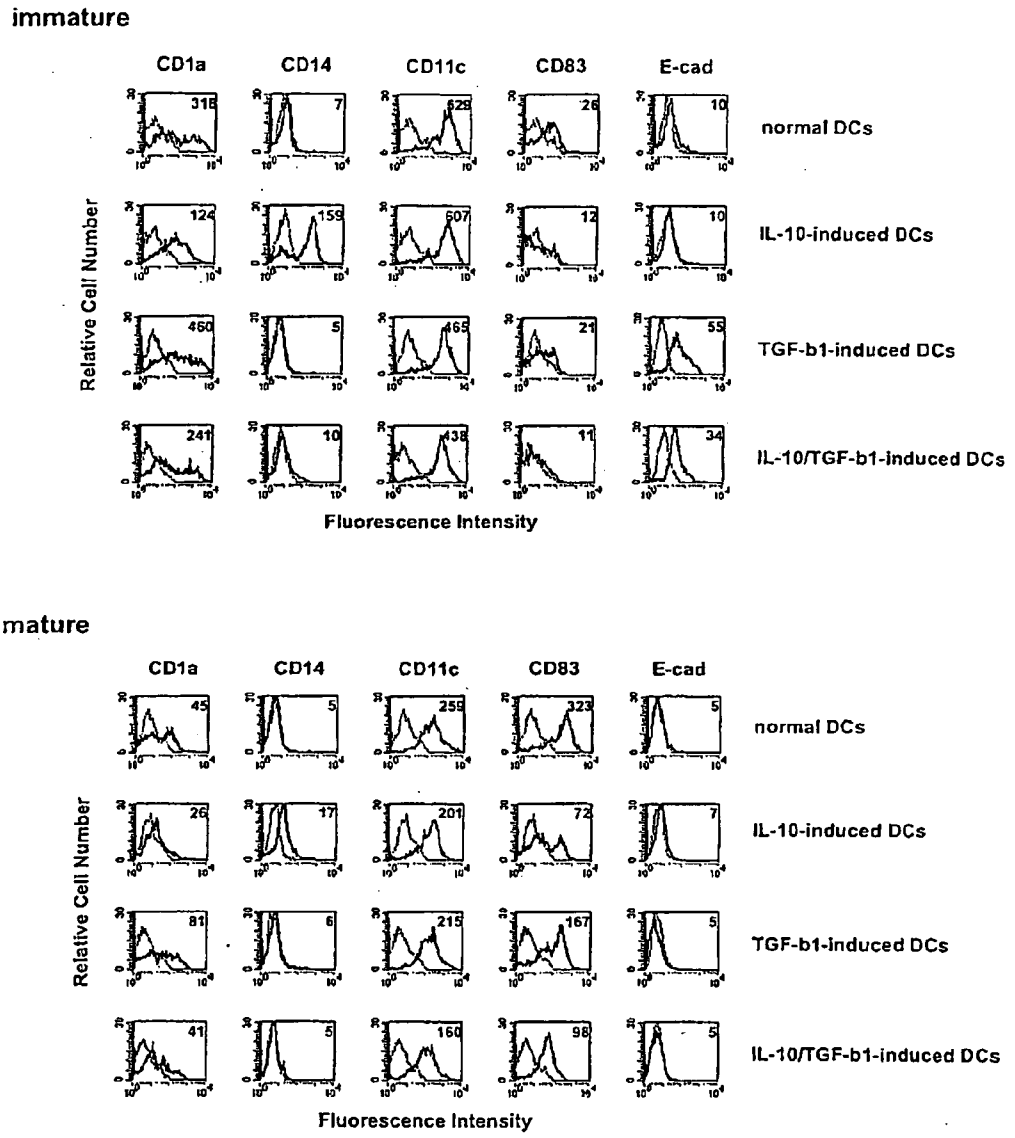
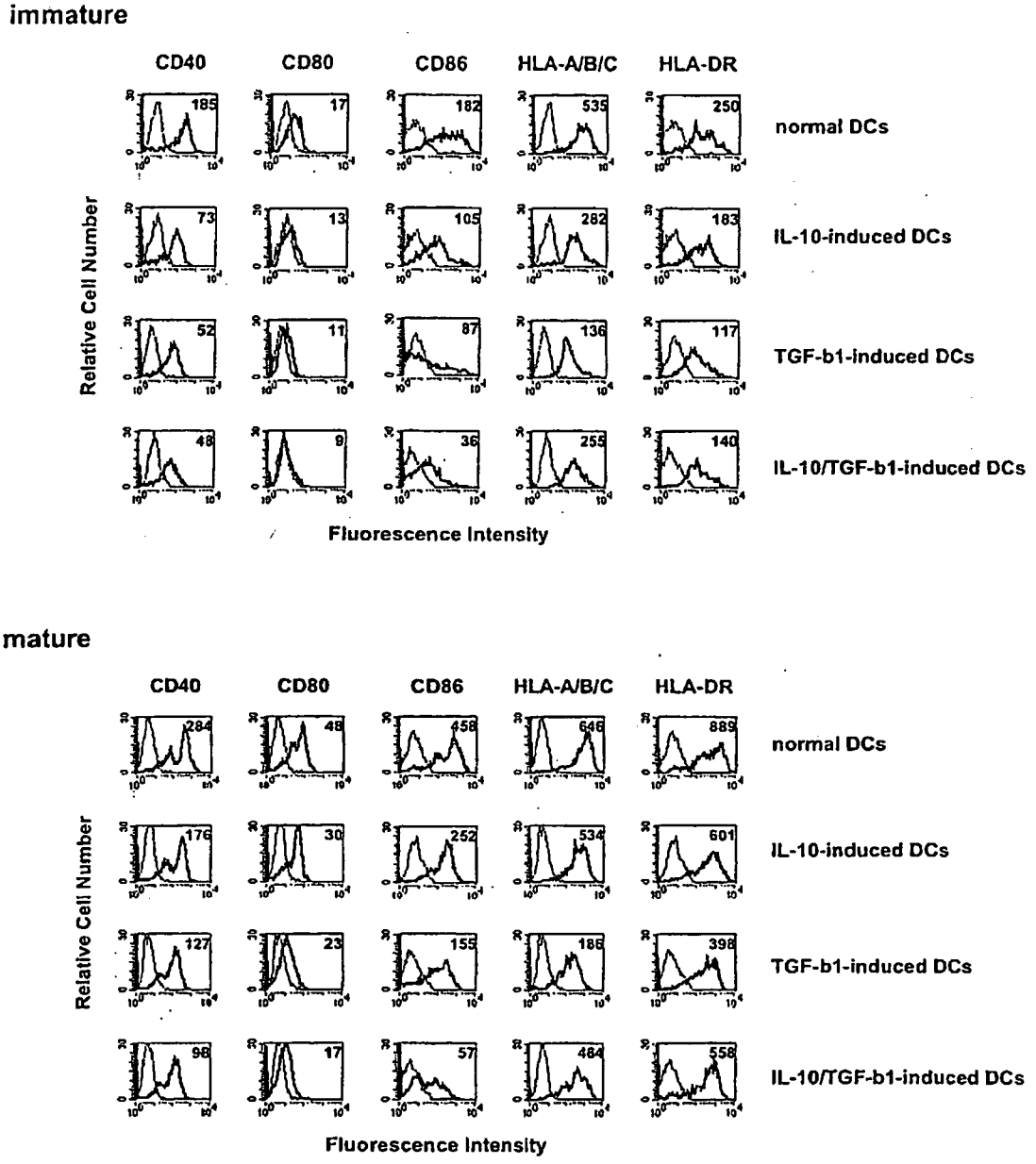

immature
CD1a
CD14
CD11c
CD83
E-cad
318
7
628
26
10
normal DCs
124
159
607
12
10
IL-10-induced DCs
460
5
465
21
55
TGF-b1-induced DCs
241
10
438
11
34
IL-10/TGF-b1-induced DCs
Relative Cell Number
Fluorescence Intensity
mature
CD1a
CD14
CD11c
CD83
E-cad
45
5
259
323
5
normal DCs
26
17
201
72
7
IL-10-induced DCs
81
6
215
167
5
TGF-b1-induced DCs
41
5
160
98
5
IL-10/TGF-b1-induced DCs
Relative Cell Number
Fluorescence Intensity

Fig. 1B


immature
CD40
CD80
CD86
HLA-A/B/C
HLA-DR
185
17
182
535
250
normal DCs
73
13
105
282
183
IL-10-induced DCs
52
11
87
136
117
TGF-b1-induced DCs
48
9
36
255
140
IL-10/TGF-b1-induced DCs
Relative Cell Number
Fluorescence Intensity
mature
CD40
CD80
CD86
HLA-A/B/C
HLA-DR
284
48
458
646
889
normal DCs
176
30
252
534
601
IL-10-induced DCs
127
23
155
186
398
TGF-b1-induced DCs
98
17
57
464
558
IL-10/TGF-b1-induced DCs
Relative Cell Number
Fluorescence Intensity

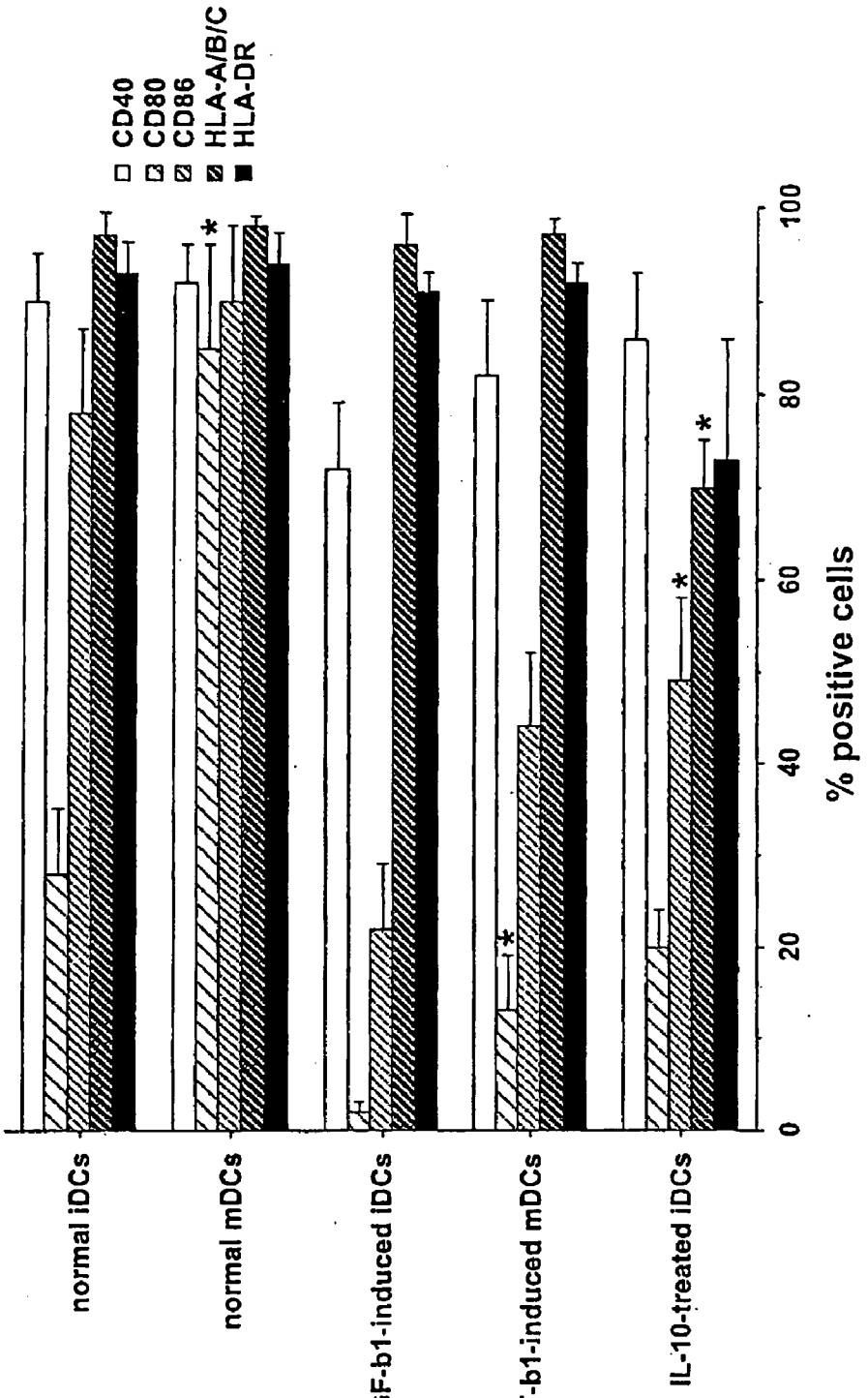
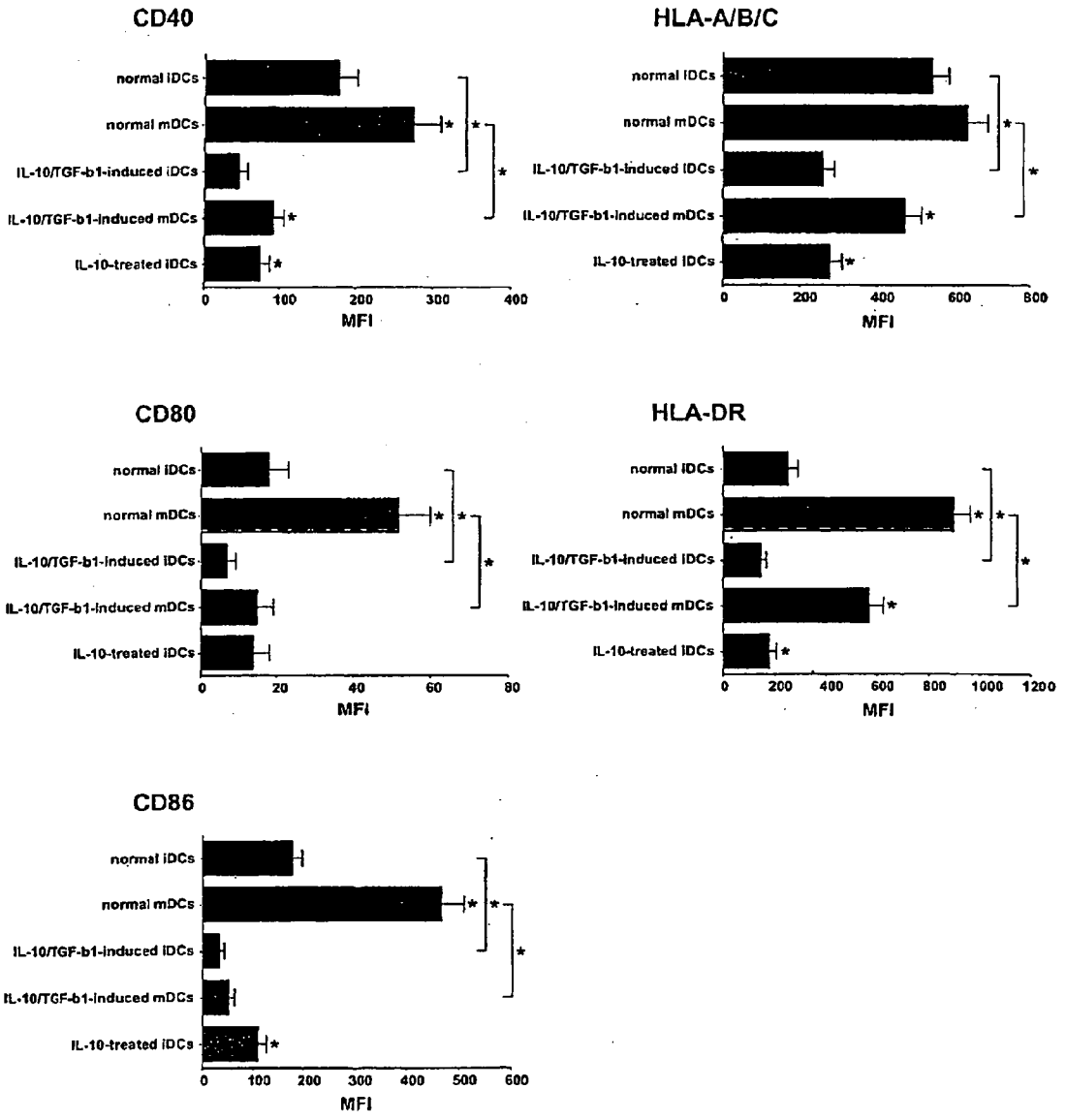

CD40
CD80
CD86
HLA-A/B/C
HLA-DR
% positive cells
0
20
40
60
80
100
normal iDCs
normal mDCs
IL-10/TGF-b1-induced iDCs
IL-10/TGF-b1-induced mDCs
IL-10-treated iDCs
*

Fig. 1C

Fig. 1D


CD40
normal iDCs
normal mDCs
IL-10/TGF-b1-induced iDCs
IL-10/TGF-b1-induced mDCs
IL-10-treated iDCs
0
100
200
300
400
MFI
HLA-A/B/C
normal iDCs
normal mDCs
IL-10/TGF-b1-induced iDCs
IL-10/TGF-b1-induced mDCs
IL-10-treated iDCs
0
200
400
600
800
MFI
CD80
normal iDCs
normal mDCs
IL-10/TGF-b1-induced iDCs
IL-10/TGF-b1-induced mDCs
IL-10-treated iDCs
0
20
40
60
80
MFI
HLA-DR
normal iDCs
normal mDCs
IL-10/TGF-b1-induced iDCs
IL-10/TGF-b1-induced mDCs
IL-10-treated iDCs
0
200
400
600
800
1000
1200
MFI
CD86
normal iDCs
normal mDCs
IL-10/TGF-b1-induced iDCs
IL-10/TGF-b1-induced mDCs
IL-10-treated iDCs
0
100
200
300
400
500
600
MFI

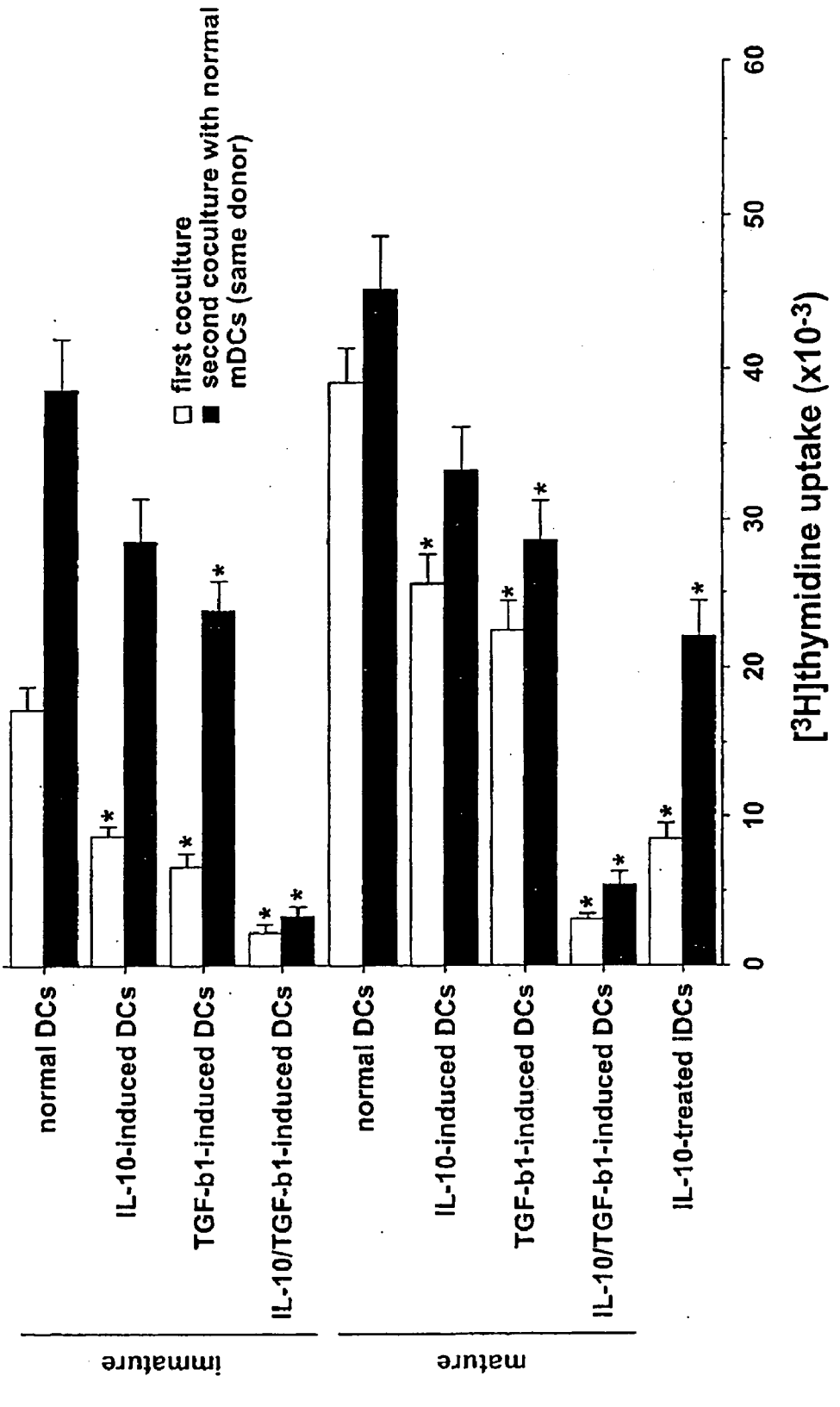

first coculture
second coculture with normal mDCs (same donor)
[3H]thymidine uptake (x10-3)
0
10
20
30
40
50
60
normal DCs
IL-10-induced DCs
TGF-b1-induced DCs
IL-10/TGF-b1-induced DCs
normal DCs
IL-10-induced DCs
TGF-b1-induced DCs
IL-10/TGF-b1-induced DCs
IL-10-treated IDCs
immature
mature

Fig. 2A

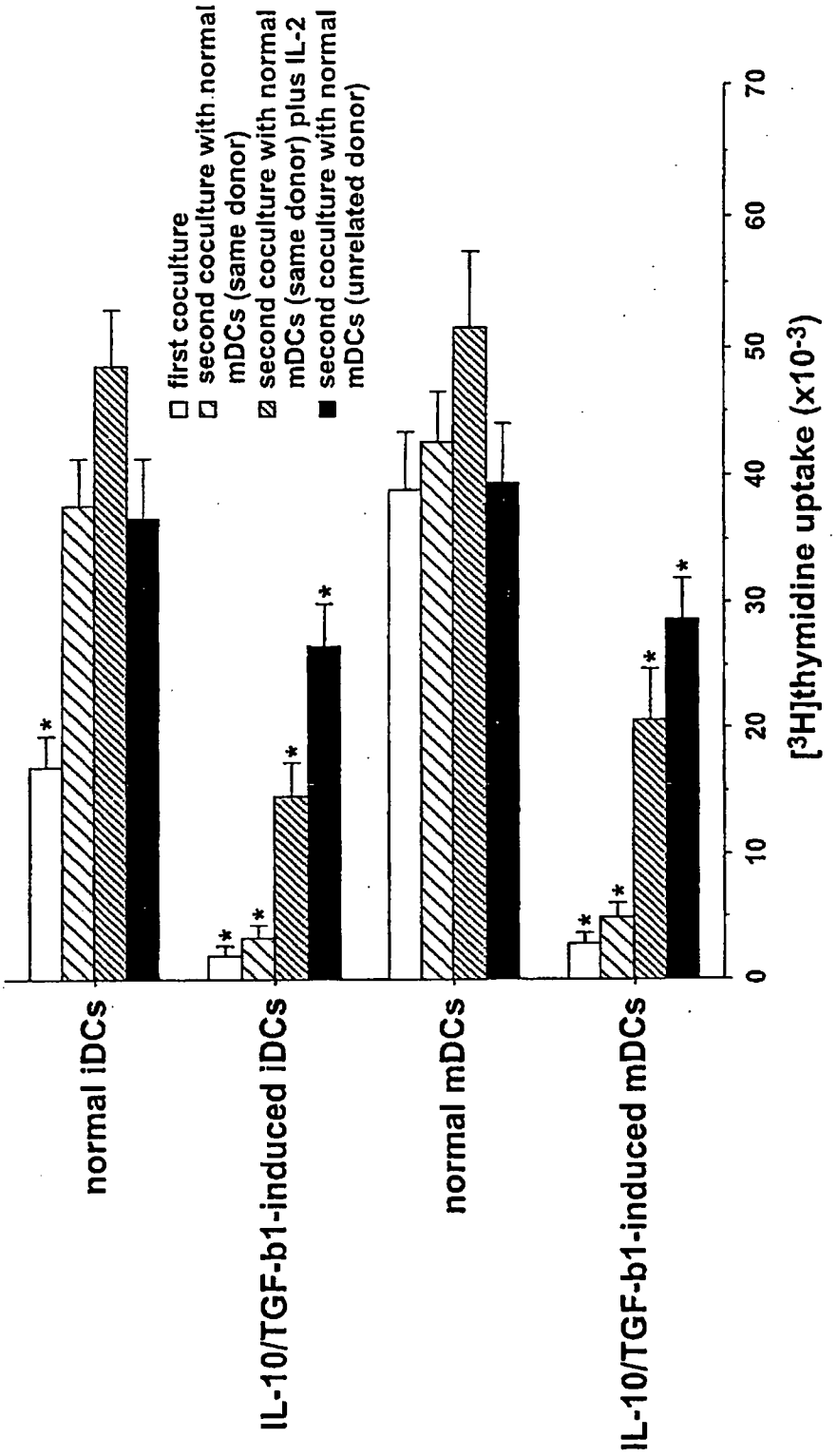

first coculture
second coculture with normal mDCs (same donor)
second coculture with normal mDCs (same donor) plus IL-2
second coculture with normal mDCs (unrelated donor)
normal iDCs
IL-10/TGF-b1-induced iDCs
normal mDCs
IL-10/TGF-b1-induced mDCs
[3H]thymidine uptake (x10-3)
0
10
20
30
40
50
60
70
*

Fig. 2B

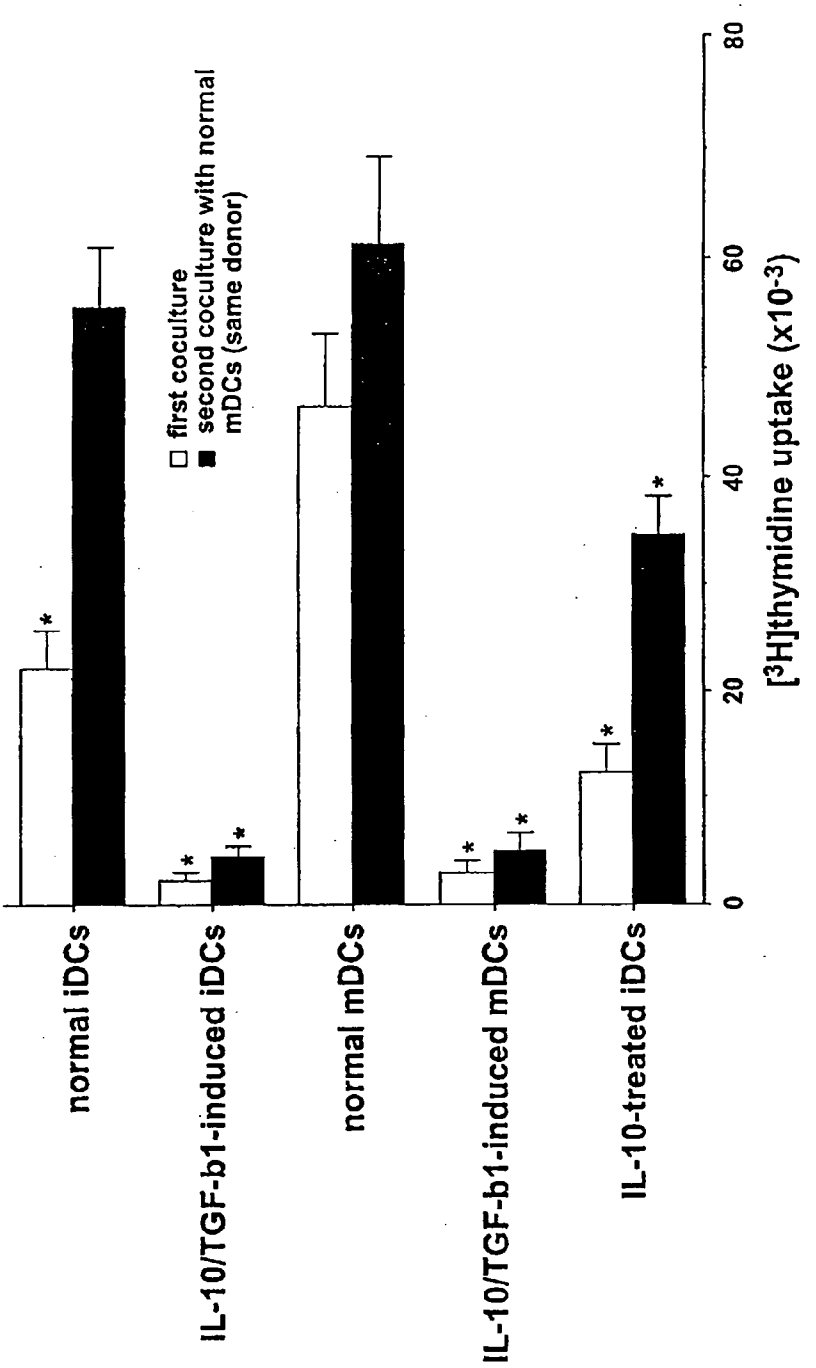
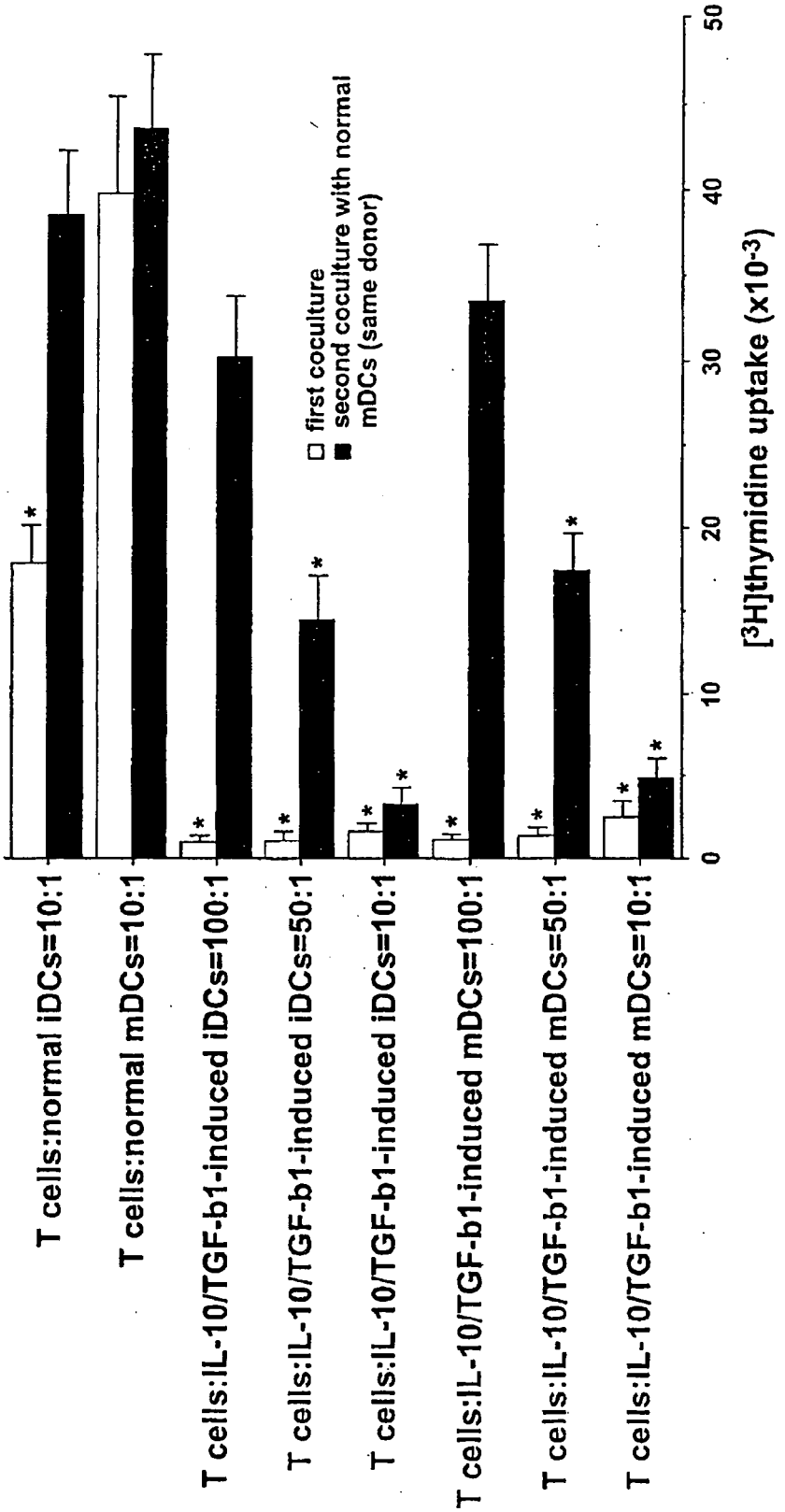

first coculture
second coculture with normal mDCs (same donor)
[3H]thymidine uptake (x10-3)
0
20
40
60
80
normal iDCs
IL-10/TGF-b1-induced iDCs
normal mDCs
IL-10/TGF-b1-induced mDCs
IL-10-treated iDCs

Fig. 2C

Fig. 2D


T cells:normal iDCs=10:1
T cells:normal mDCs=10:1
T cells:IL-10/TGF-b1-induced iDCs=100:1
T cells:IL-10/TGF-b1-induced iDCs=50:1
T cells:IL-10/TGF-b1-induced iDCs=10:1
T cells:IL-10/TGF-b1-induced mDCs=100:1
T cells:IL-10/TGF-b1-induced mDCs=50:1
T cells:IL-10/TGF-b1-induced mDCs=10:1
first coculture
second coculture with normal mDCs (same donor)
[3H]thymidine uptake (x10-3)
0
10
20
30
40
50
*

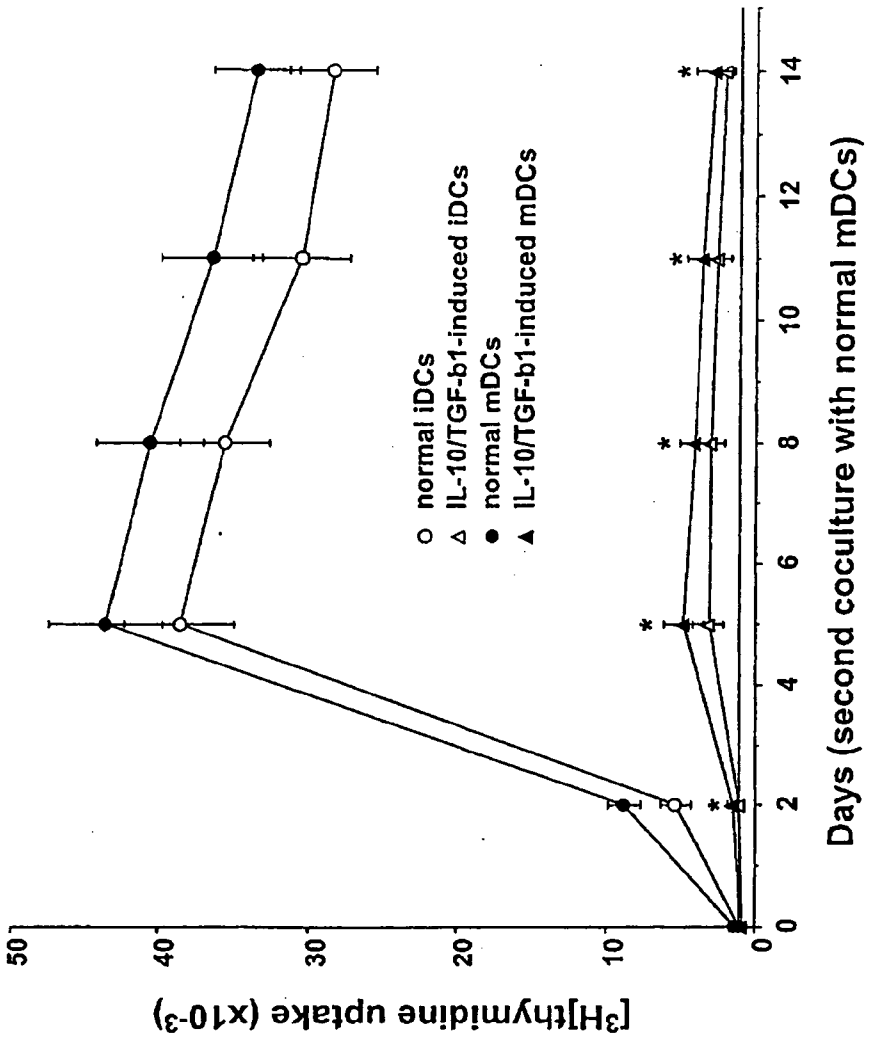

[3H]thymidine uptake (x10-3)
50
40
30
20
10
0
0
2
4
6
8
10
12
14
Days (second coculture with normal mDCs)
normal iDCs
IL-10/TGF-b1-induced iDCs
normal mDCs
IL-10/TGF-b1-induced mDCs
*

Fig. 2E

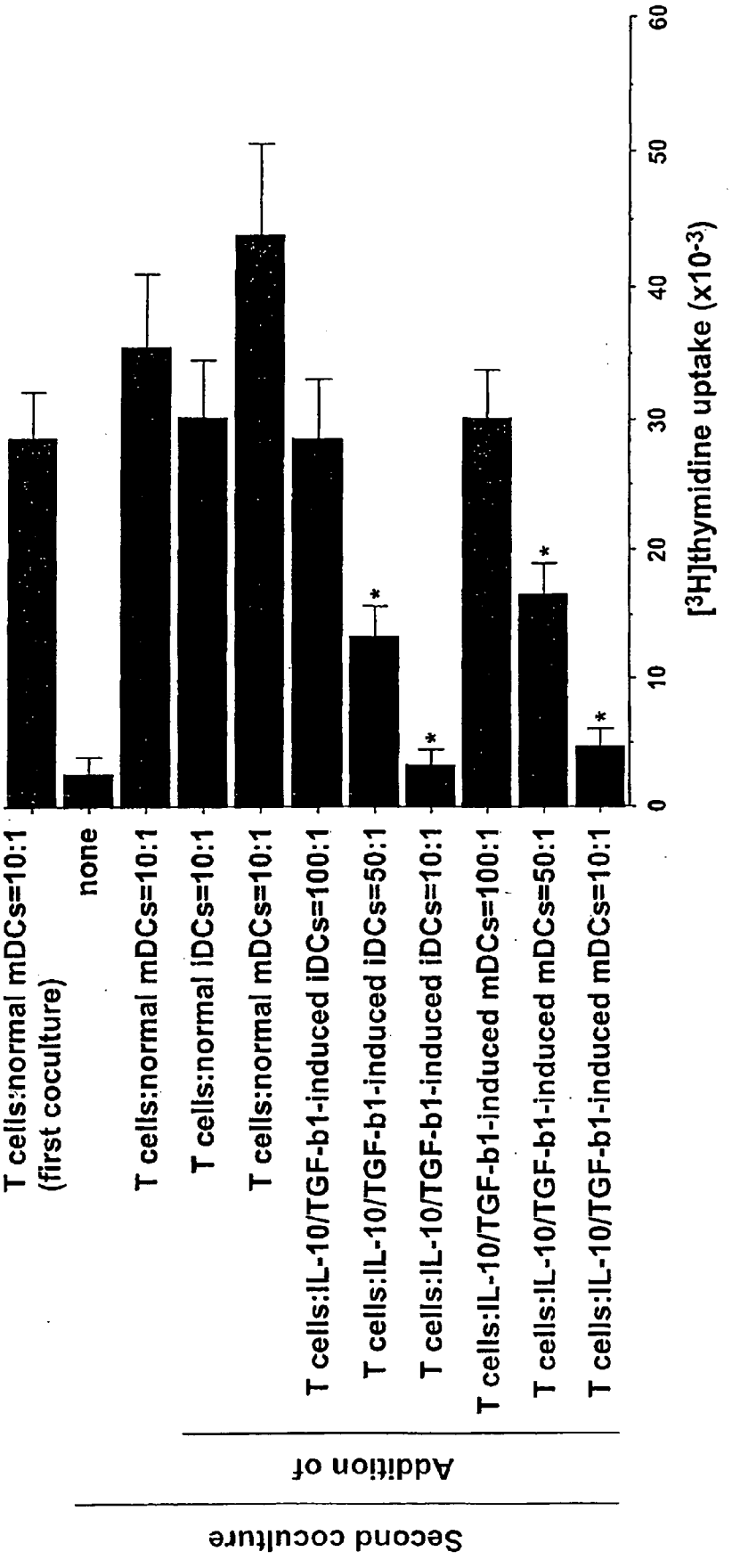
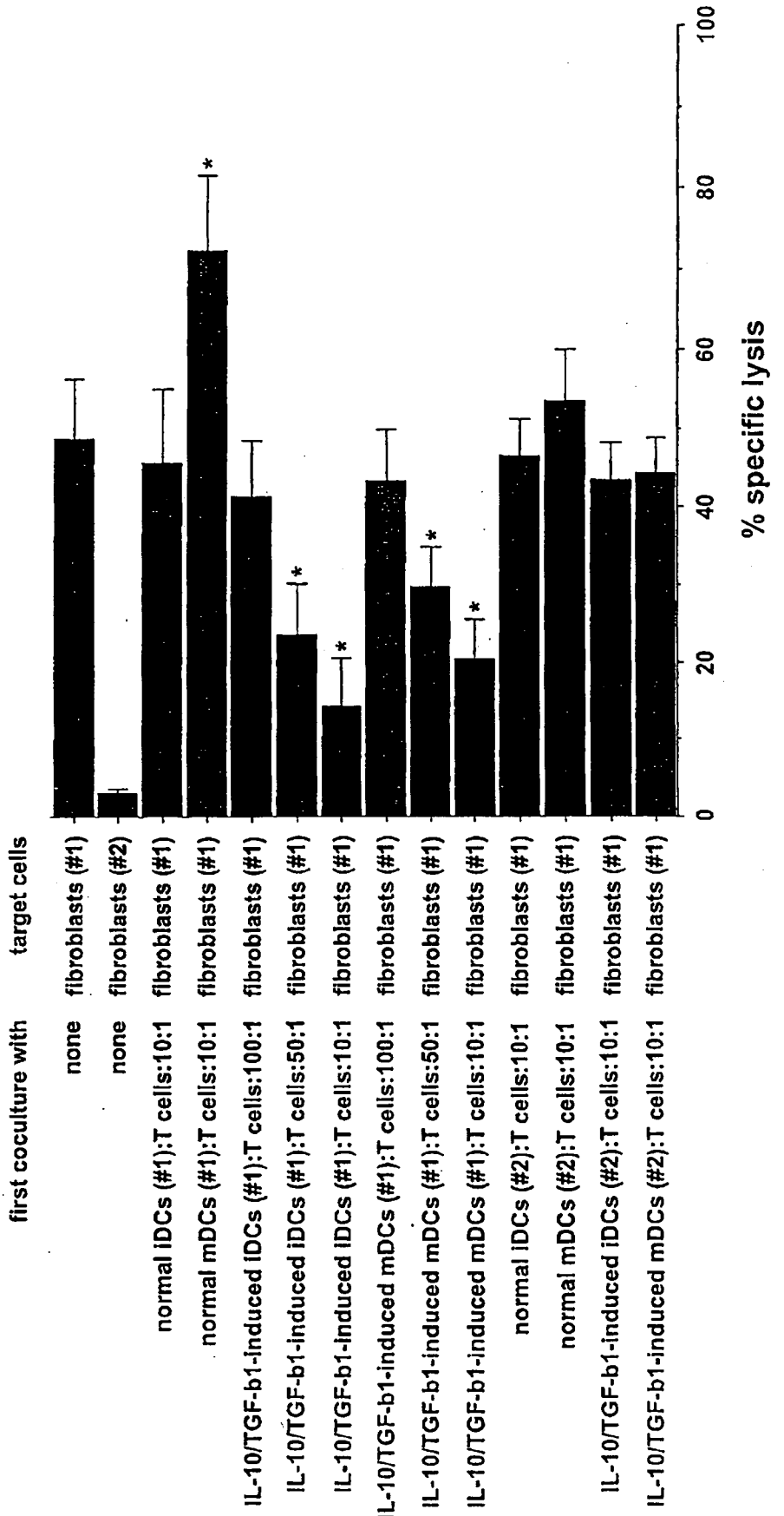

[3H]thymidine uptake (x10-3)
0
10
20
30
40
50
60
Second coculture
Addition of
T cells:normal mDCs=10:1 (first coculture)
none
T cells:normal mDCs=10:1
T cells:normal iDCs=10:1
T cells:normal mDCs=10:1
T cells:IL-10/TGF-b1-induced iDCs=100:1
T cells:IL-10/TGF-b1-induced iDCs=50:1
T cells:IL-10/TGF-b1-induced iDCs=10:1
T cells:IL-10/TGF-b1-induced mDCs=100:1
T cells:IL-10/TGF-b1-induced mDCs=50:1
T cells:IL-10/TGF-b1-induced mDCs=10:1
*
*
*
*

Fig. 2F

Fig. 2G


first coculture with
target cells
none
fibroblasts (#1)
none
fibroblasts (#2)
normal iDCs (#1):T cells:10:1
fibroblasts (#1)
normal mDCs (#1):T cells:10:1
fibroblasts (#1)
IL-10/TGF-b1-induced iDCs (#1):T cells:100:1
fibroblasts (#1)
IL-10/TGF-b1-induced iDCs (#1):T cells:50:1
fibroblasts (#1)
IL-10/TGF-b1-induced iDCs (#1):T cells:10:1
fibroblasts (#1)
IL-10/TGF-b1-induced mDCs (#1):T cells:100:1
fibroblasts (#1)
IL-10/TGF-b1-induced mDCs (#1):T cells:50:1
fibroblasts (#1)
IL-10/TGF-b1-induced mDCs (#1):T cells:10:1
fibroblasts (#1)
normal iDCs (#2):T cells:10:1
fibroblasts (#1)
normal mDCs (#2):T cells:10:1
fibroblasts (#1)
IL-10/TGF-b1-induced iDCs (#2):T cells:10:1
fibroblasts (#1)
IL-10/TGF-b1-induced mDCs (#2):T cells:10:1
fibroblasts (#1)
0
20
40
60
80
100
% specific lysis

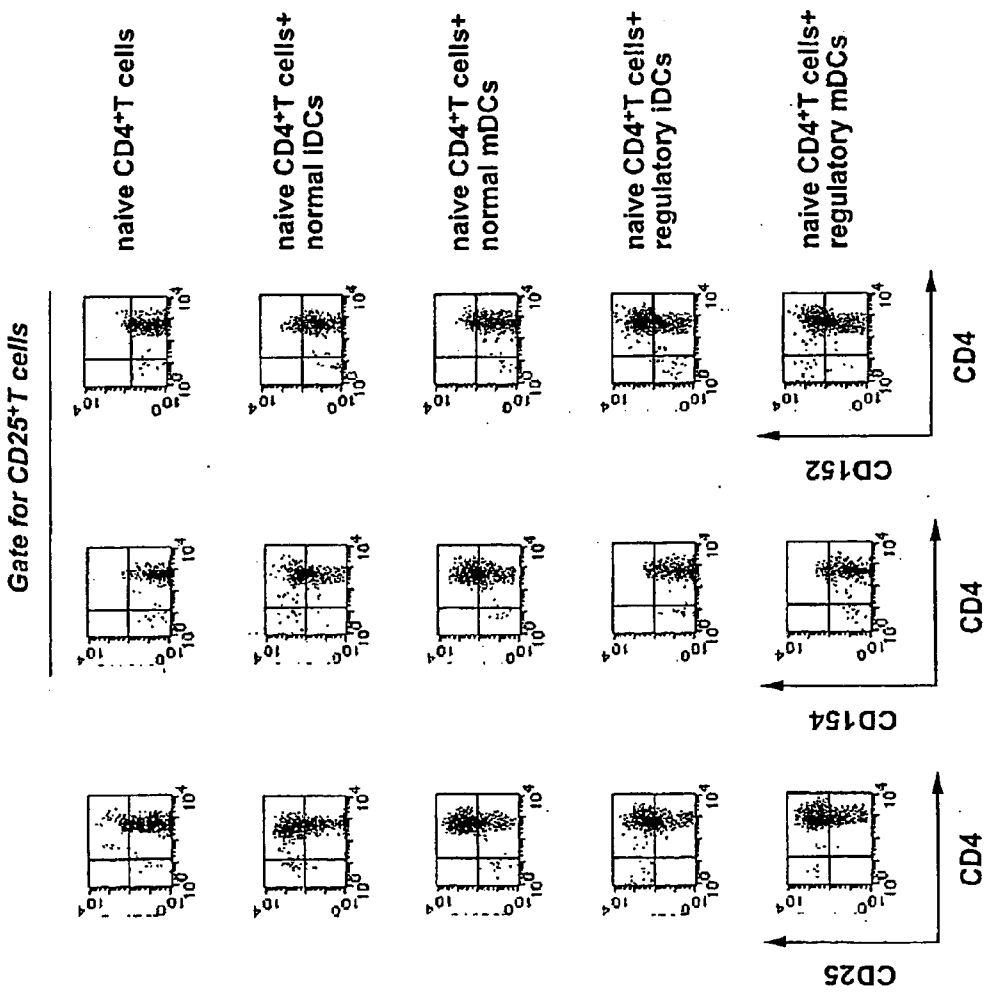

naive CD4+T cells
naive CD4+T cells+ normal IDCs
naive CD4+T cells+ normal mDCs
naive CD4+T cells+ regulatory iDCs
naive CD4+T cells+ regulatory mDCs
Gate for CD25+T cells
CD4
CD152
CD4
CD154
CD4
CD25

Fig. 3A

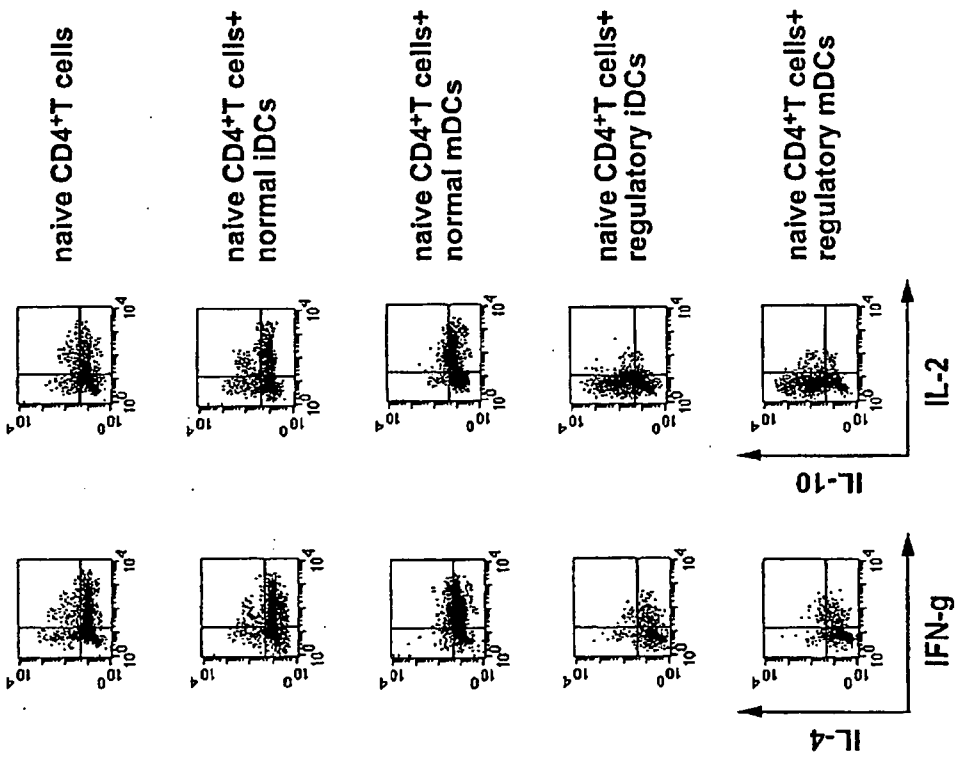

naive CD4+T cells
naive CD4+T cells+ normal iDCs
naive CD4+T cells+ normal mDCs
naive CD4+T cells+ regulatory iDCs
naive CD4+T cells+ regulatory mDCs
IL-2
IL-10
IFN-g
IL-4

Fig. 3B

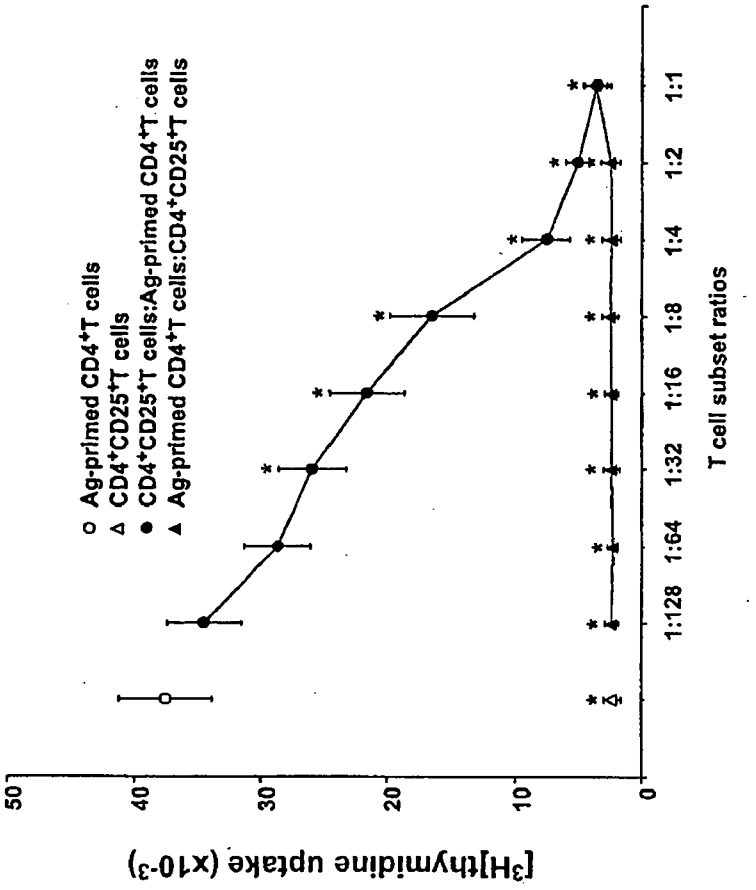
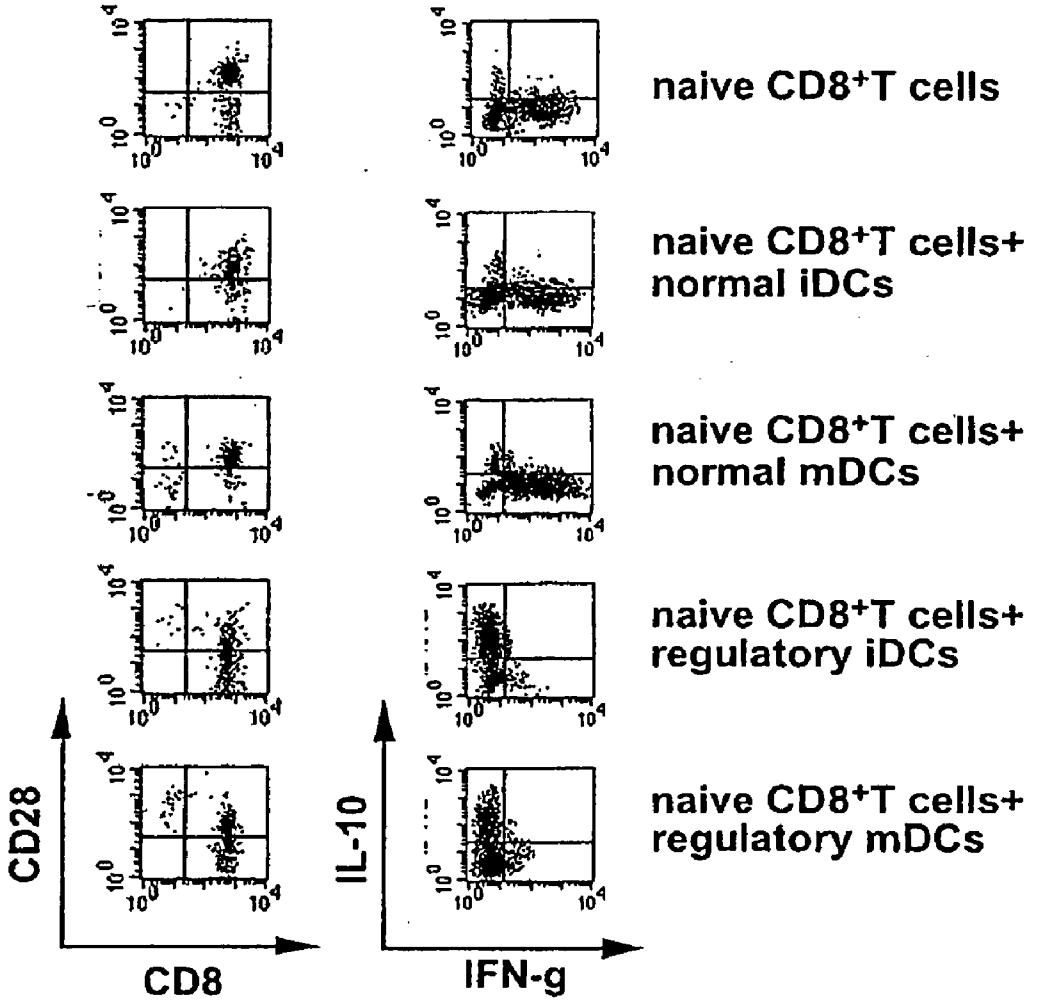

o Ag-primed CD4+T cells
Δ CD4+CD25+T cells
● CD4+CD25+T cells:Ag-primed CD4+T cells
▲ Ag-primed CD4+T cells:CD4+CD25+T cells
[3H]thymidine uptake (x10-3)
50
40
30
20
10
0
1:128
1:64
1:32
1:16
1:8
1:4
1:2
1:1
T cell subset ratios

Fig. 3C

Fig. 4A


naive CD8+T cells
naive CD8+T cells+ normal iDCs
naive CD8+T cells+ normal mDCs
naive CD8+T cells+ regulatory iDCs
naive CD8+T cells+ regulatory mDCs
CD28
CD8
IL-10
IFN-g

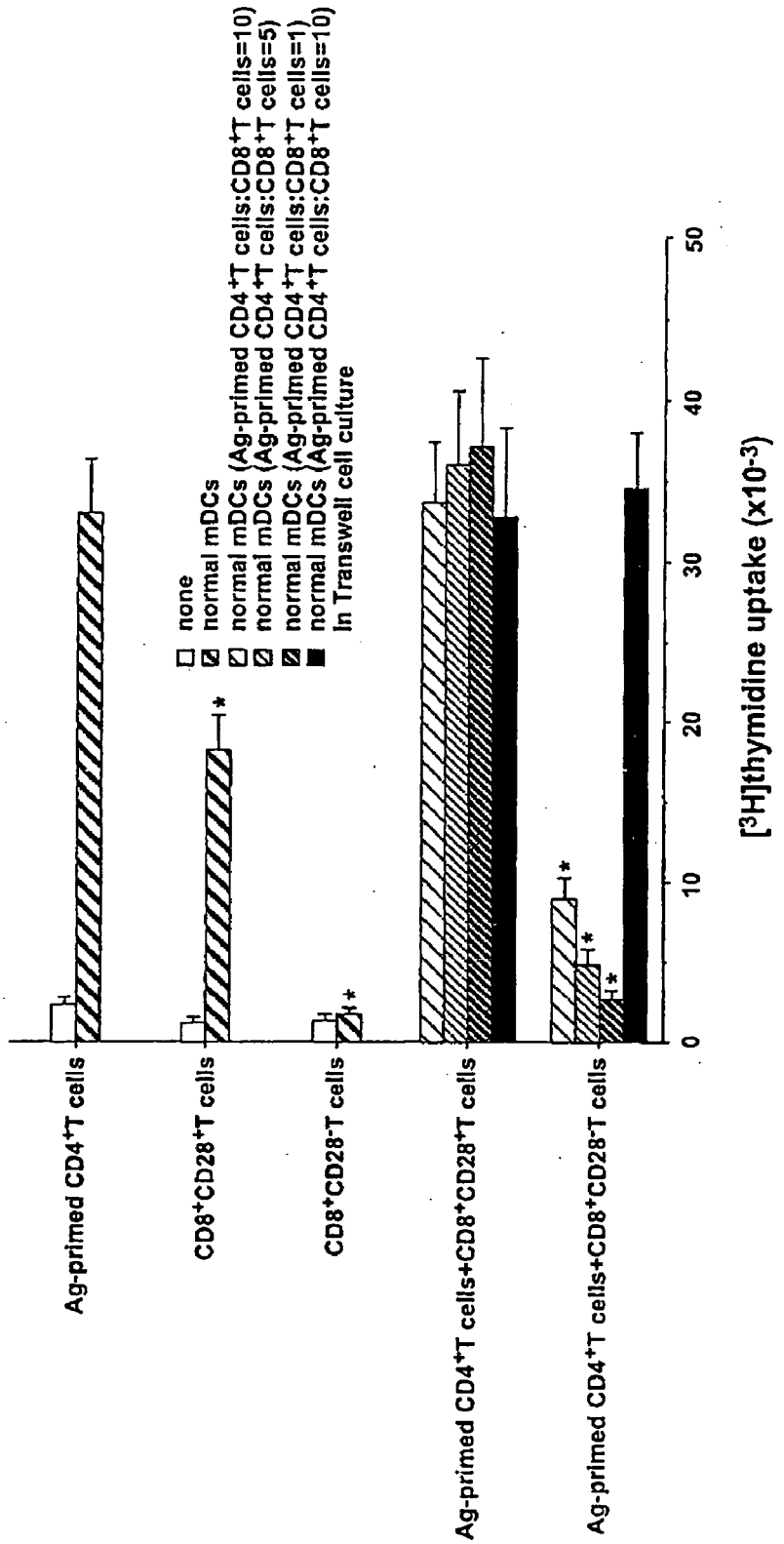

none
normal mDCs
normal mDCs (Ag-primed CD4+T cells:CD8+T cells=10)
normal mDCs (Ag-primed CD4+T cells:CD8+T cells=5)
normal mDCs (Ag-primed CD4+T cells:CD8+T cells=1)
normal mDCs (Ag-primed CD4+T cells:CD8+T cells=10)
In Transwell cell culture
Ag-primed CD4+T cells
CD8+CD28+T cells
CD8+CD28-T cells
Ag-primed CD4+T cells+CD8+CD28+T cells
Ag-primed CD4+T cells+CD8+CD28-T cells
[3H]thymidine uptake (x10-3)
0
10
20
30
40
50

Fig. 4B

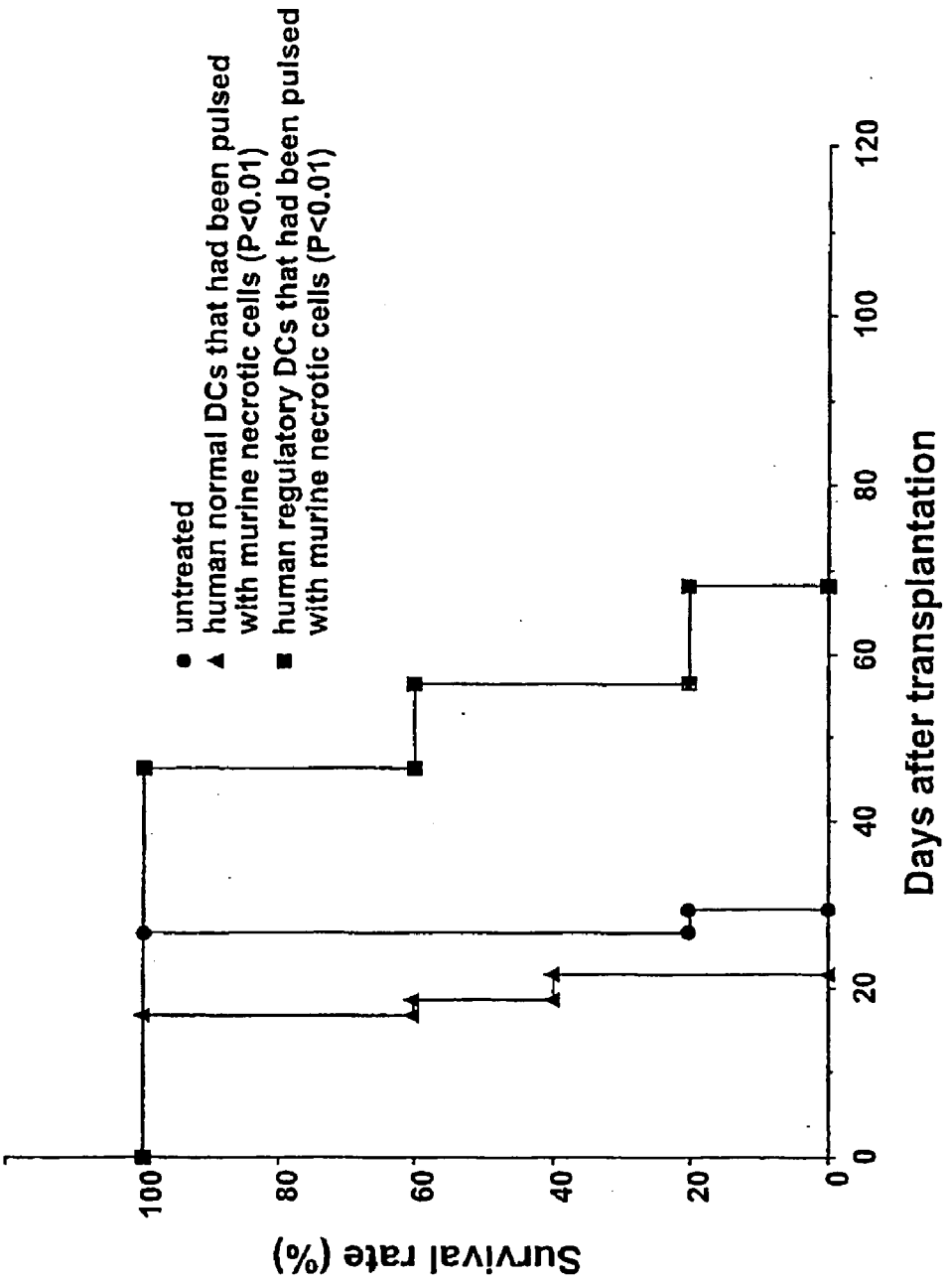

untreated
human normal DCs that had been pulsed with murine necrotic cells (P<0.01)
human regulatory DCs that had been pulsed with murine necrotic cells (P<0.01)
Survival rate (%)
100
80
60
40
20
0
0
20
40
60
80
100
120
Days after transplantation

Fig. 5A

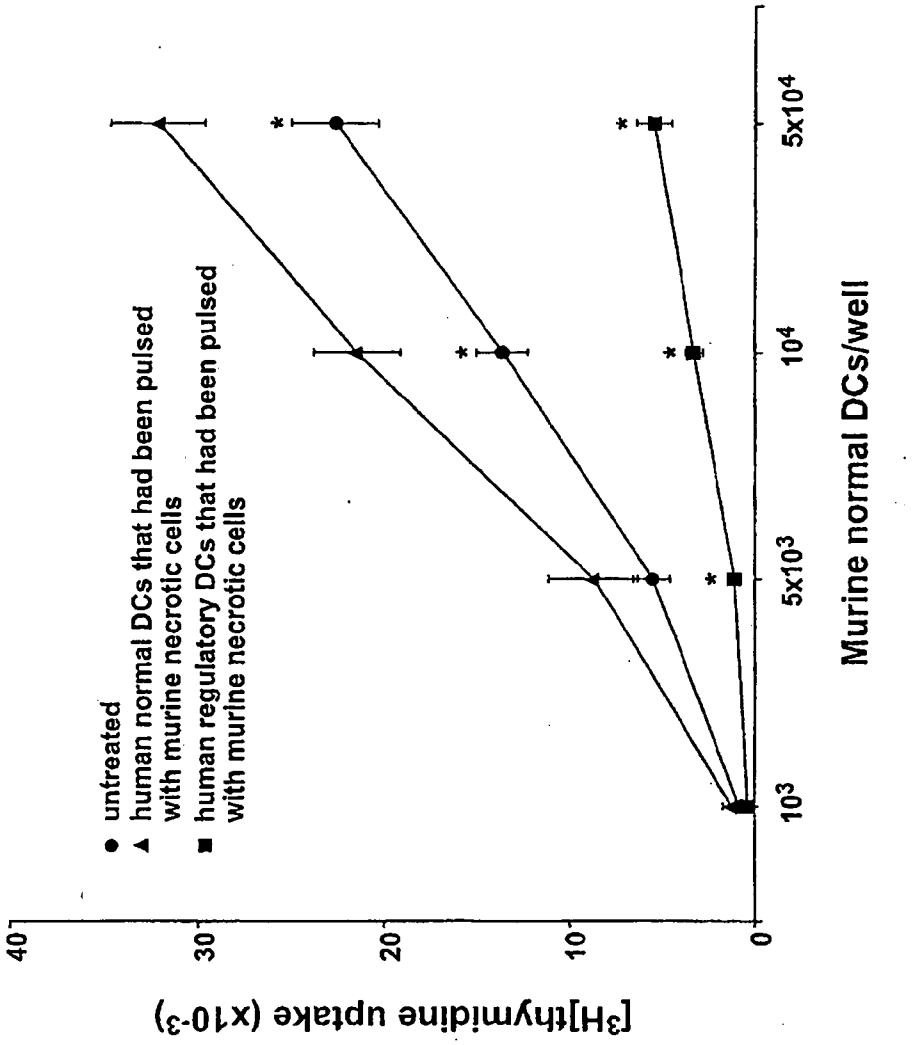

[3H]thymidine uptake (x10-3)
0
10
20
30
40
10^3
5x10^3
10^4
5x10^4
Murine normal DCs/well
untreated
human normal DCs that had been pulsed with murine necrotic cells
human regulatory DCs that had been pulsed with murine necrotic cells
*

Fig. 5B

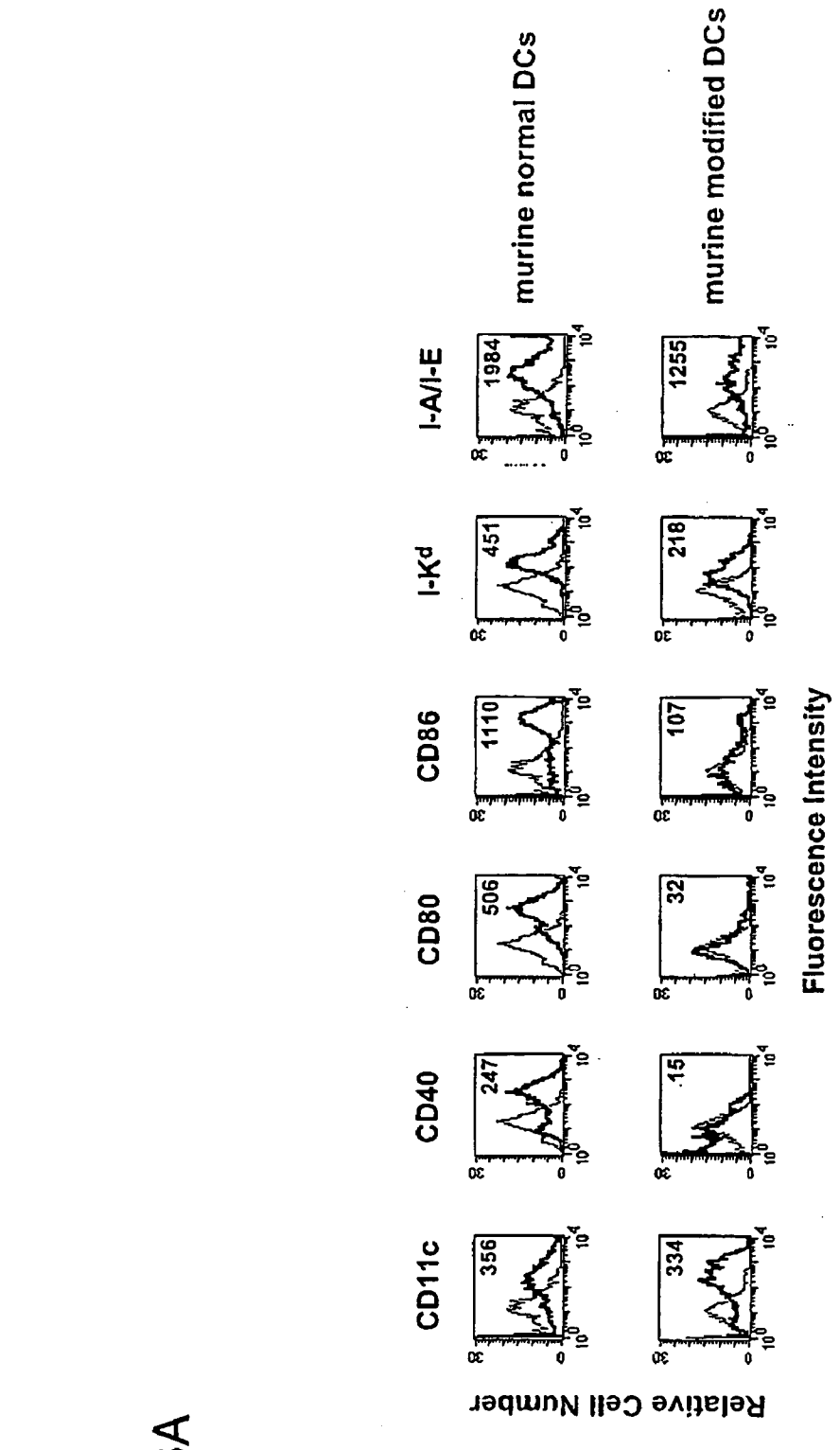

murine normal DCs
murine modified DCs
I-A/I-E
I-K^d
CD86
CD80
CD40
CD11c
1984
451
1110
506
247
356
1255
218
107
32
15
334
Relative Cell Number
Fluorescence Intensity

Fig. 6A

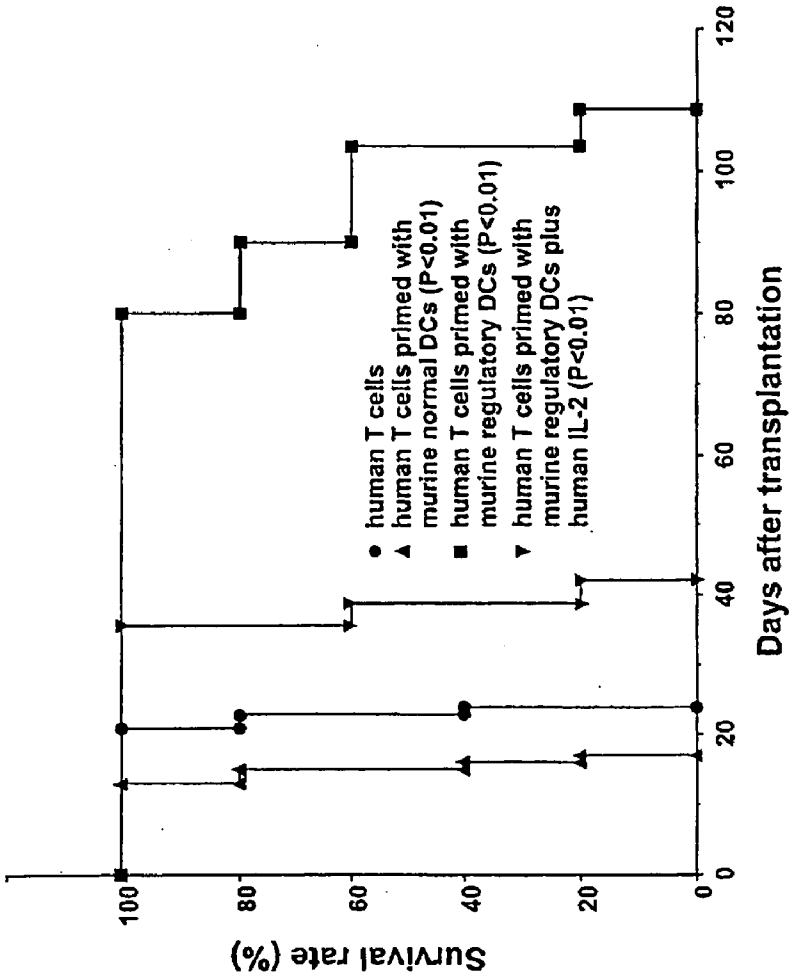

Survival rate (%)
100
80
60
40
20
0
0
20
40
60
80
100
120
Days after transplantation
human T cells
human T cells primed with murine normal DCs (P<0.01)
human T cells primed with murine regulatory DCs (P<0.01)
human T cells primed with murine regulatory DCs plus human IL-2 (P<0.01)

Fig. 6B

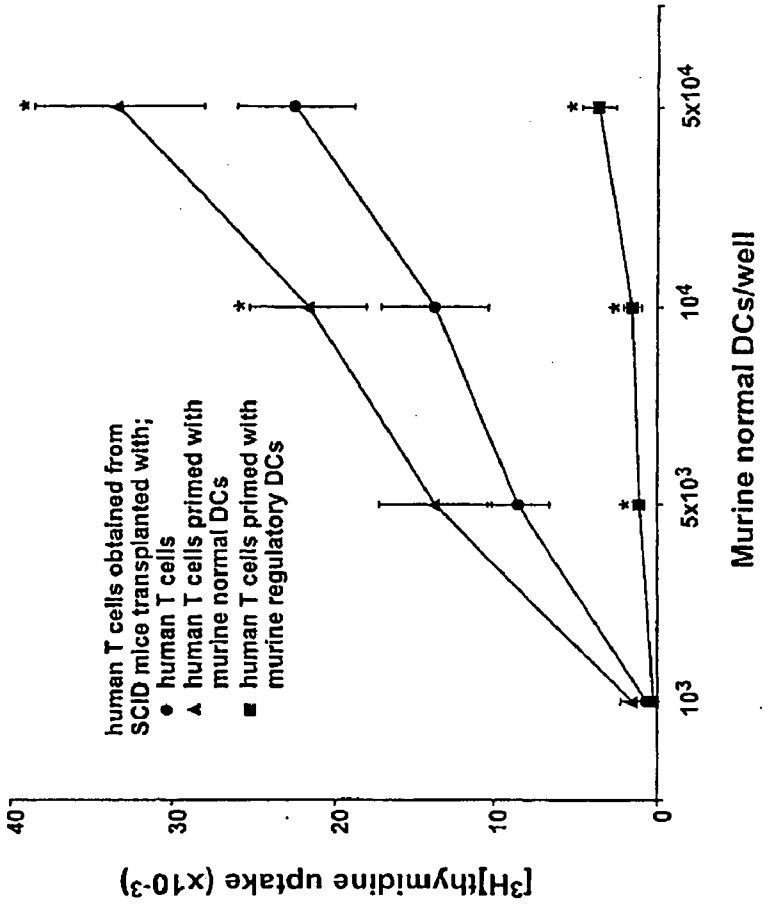

[3H]thymidine uptake (x10-3)
0
10
20
30
40
10^3
5x10^3
10^4
5x10^4
Murine normal DCs/well
human T cells obtained from SCID mice transplanted with;
● human T cells
▲ human T cells primed with murine normal DCs
■ human T cells primed with murine regulatory DCs

Fig. 6C

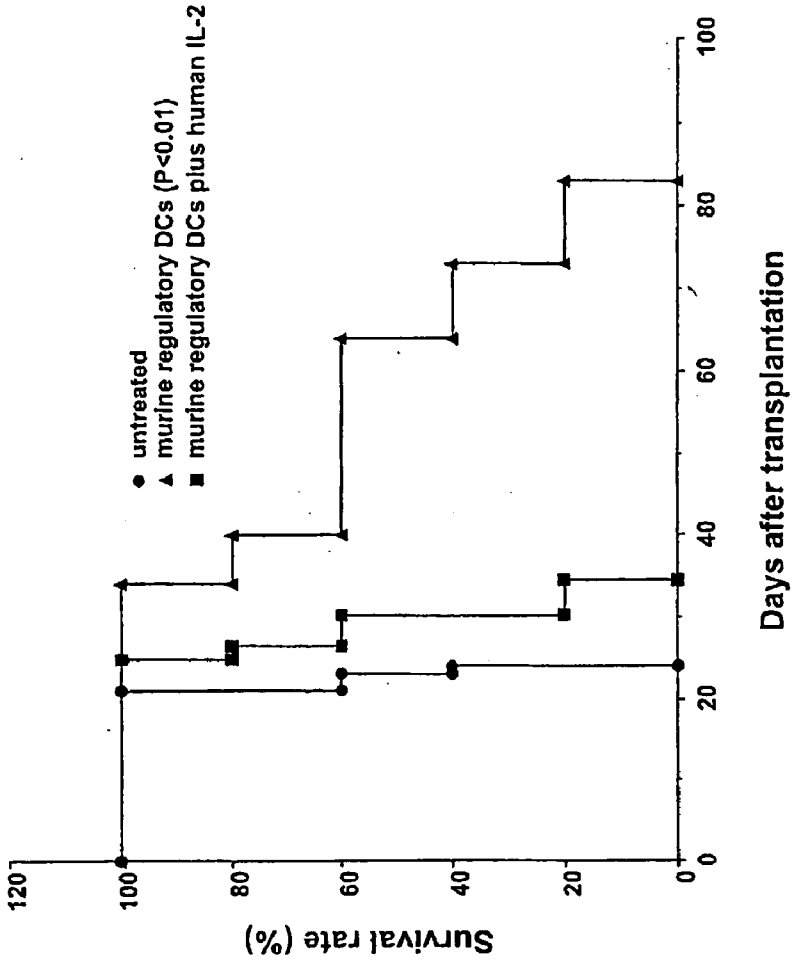

untreated
murine regulatory DCs (P<0.01)
murine regulatory DCs plus human IL-2
Survival rate (%)
120
100
80
60
40
20
0
0
20
40
60
80
100
Days after transplantation

Fig. 6D

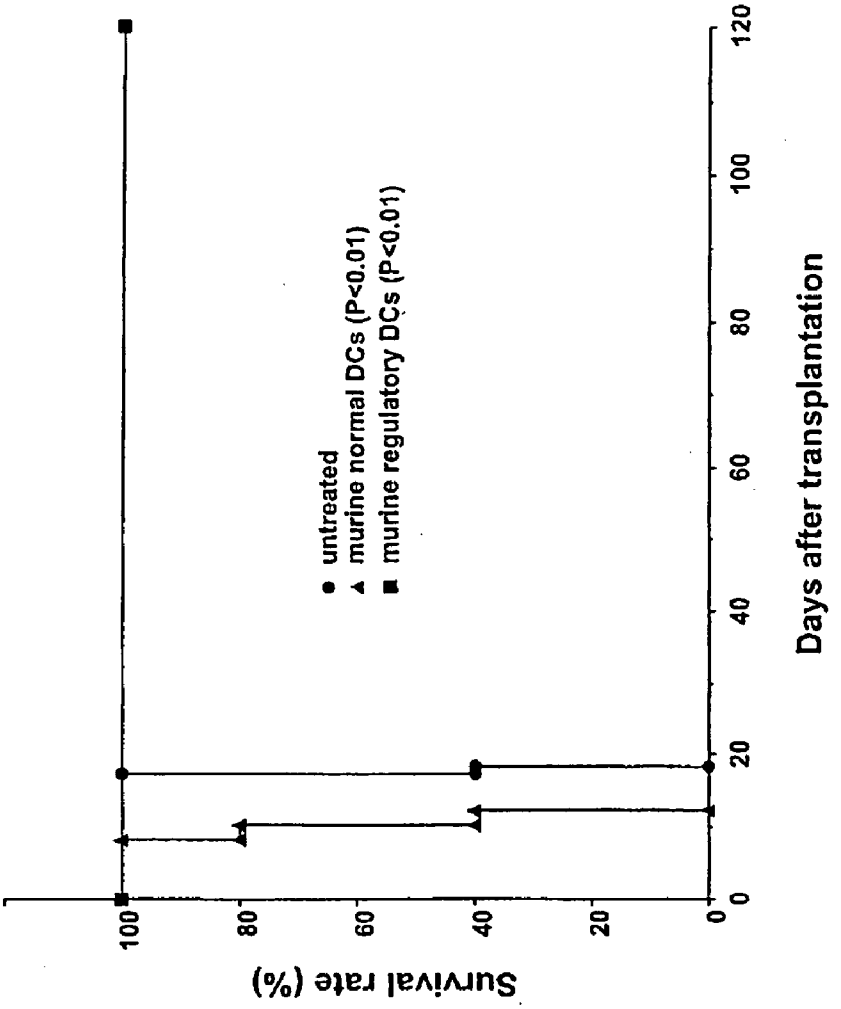

Survival rate (%)
100
80
60
40
20
0
0
20
40
60
80
100
120
Days after transplantation
untreated
murine normal DCs (P<0.01)
murine regulatory DCs (P<0.01)

Fig. 6E

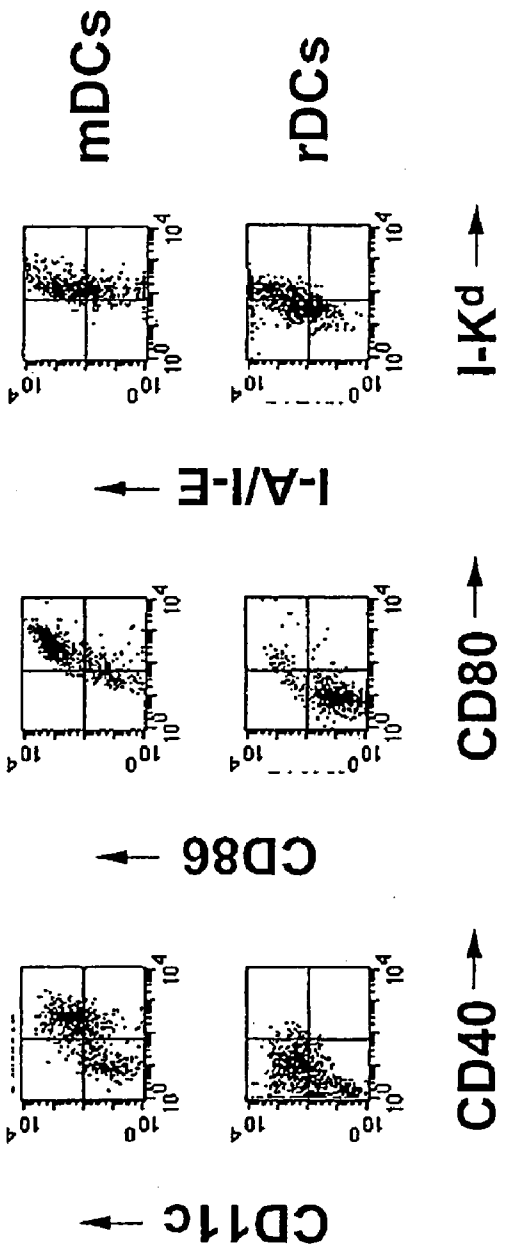


Fig. 7A

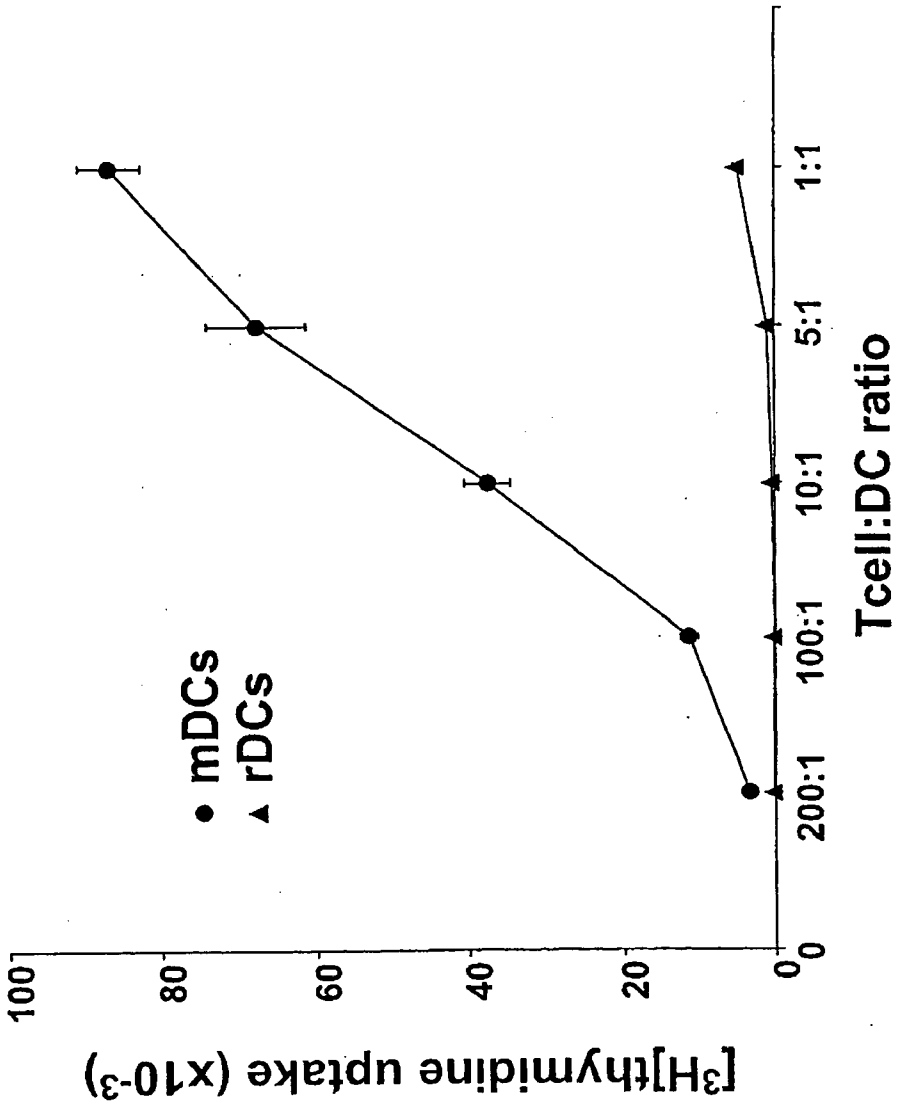

[3H]thymidine uptake (x10-3)
100
80
60
40
20
0
0
200:1
100:1
10:1
5:1
1:1
Tcell:DC ratio
mDCs
rDCs

Fig. 7B

Fig. 7C

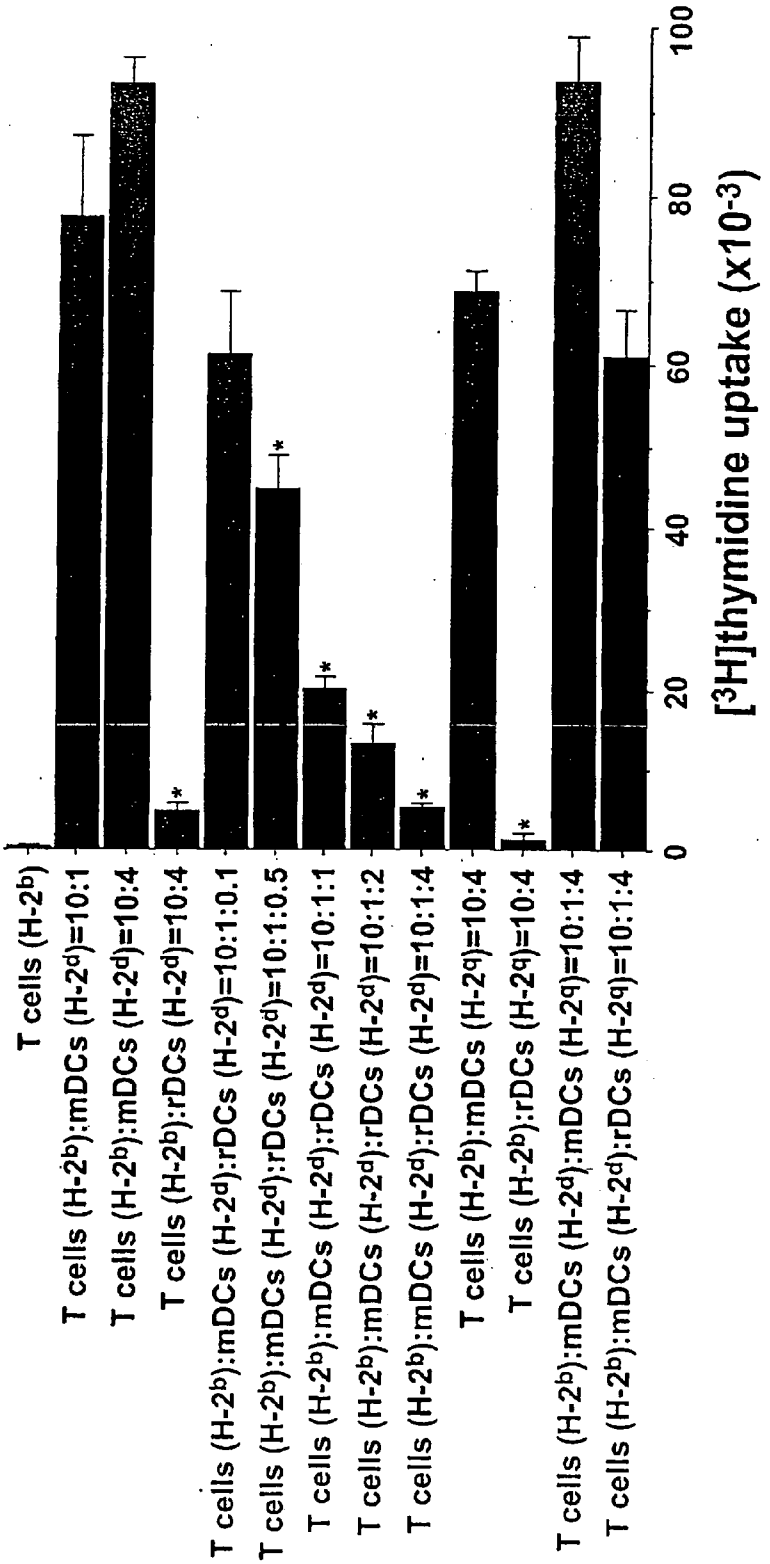
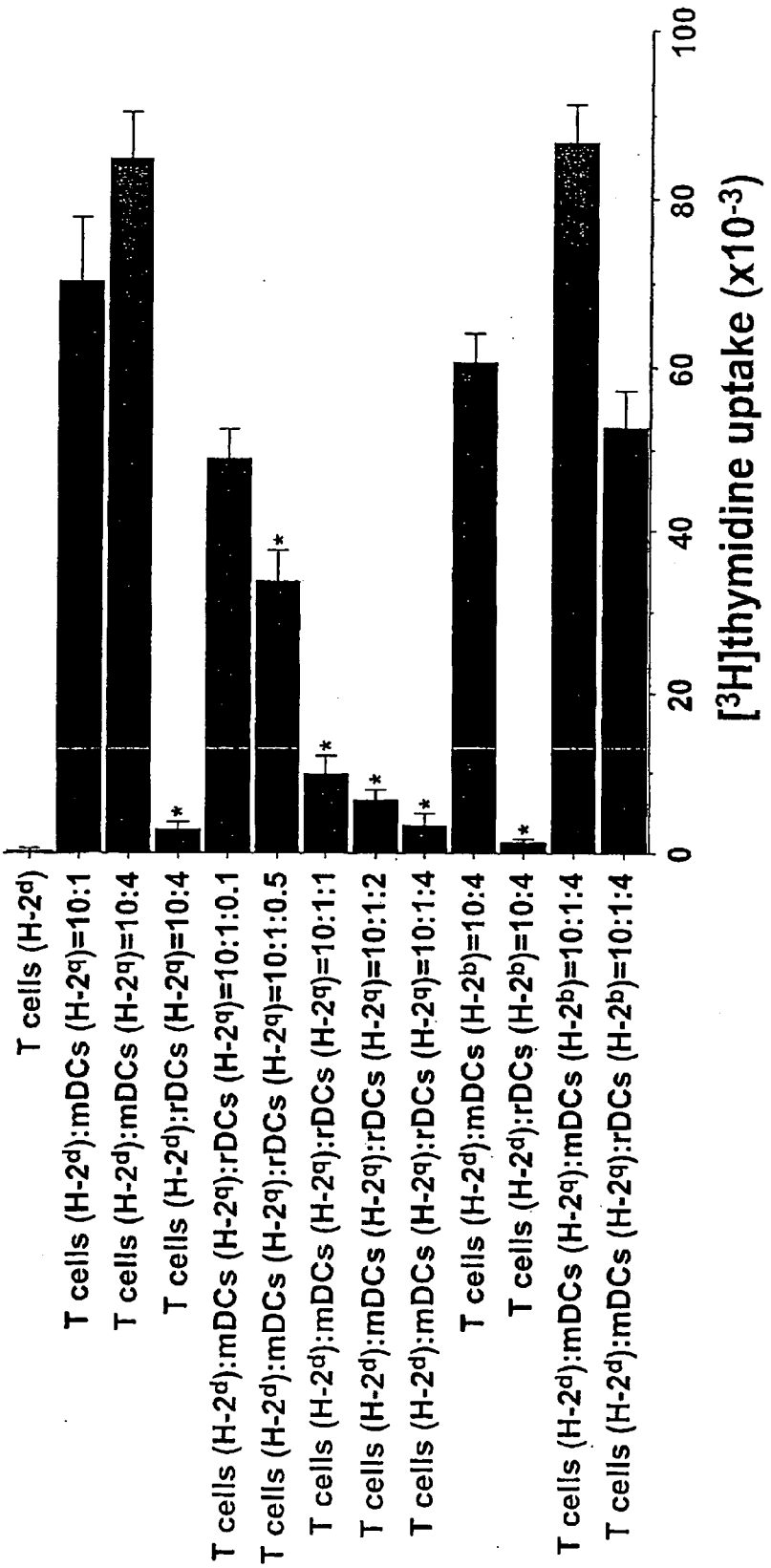

T cells (H-2b)
T cells (H-2b):mDCs (H-2d)=10:1
T cells (H-2b):mDCs (H-2d)=10:4
T cells (H-2b):rDCs (H-2d)=10:4
T cells (H-2b):mDCs (H-2d):rDCs (H-2d)=10:1:0.1
T cells (H-2b):mDCs (H-2d):rDCs (H-2d)=10:1:0.5
T cells (H-2b):mDCs (H-2d):rDCs (H-2d)=10:1:1
T cells (H-2b):mDCs (H-2d):rDCs (H-2d)=10:1:2
T cells (H-2b):mDCs (H-2d):rDCs (H-2d)=10:1:4
T cells (H-2b):mDCs (H-2q)=10:4
T cells (H-2b):rDCs (H-2q)=10:4
T cells (H-2b):mDCs (H-2d):mDCs (H-2q)=10:1:4
T cells (H-2b):mDCs (H-2d):rDCs (H-2q)=10:1:4
0
20
40
60
80
100
[3H]thymidine uptake (x10-3)

Fig. 7D


T cells (H-2d)
T cells (H-2d):mDCs (H-2q)=10:1
T cells (H-2d):mDCs (H-2q)=10:4
T cells (H-2d):rDCs (H-2q)=10:4
T cells (H-2d):mDCs (H-2q):rDCs (H-2q)=10:1:0.1
T cells (H-2d):mDCs (H-2q):rDCs (H-2q)=10:1:0.5
T cells (H-2d):mDCs (H-2q):rDCs (H-2q)=10:1:1
T cells (H-2d):mDCs (H-2q):rDCs (H-2q)=10:1:2
T cells (H-2d):mDCs (H-2q):rDCs (H-2q)=10:1:4
T cells (H-2d):mDCs (H-2b)=10:4
T cells (H-2d):rDCs (H-2b)=10:4
T cells (H-2d):mDCs (H-2q):mDCs (H-2b)=10:1:4
T cells (H-2d):mDCs (H-2q):rDCs (H-2b)=10:1:4
0
20
40
60
80
100
[3H]thymidine uptake (x10-3)

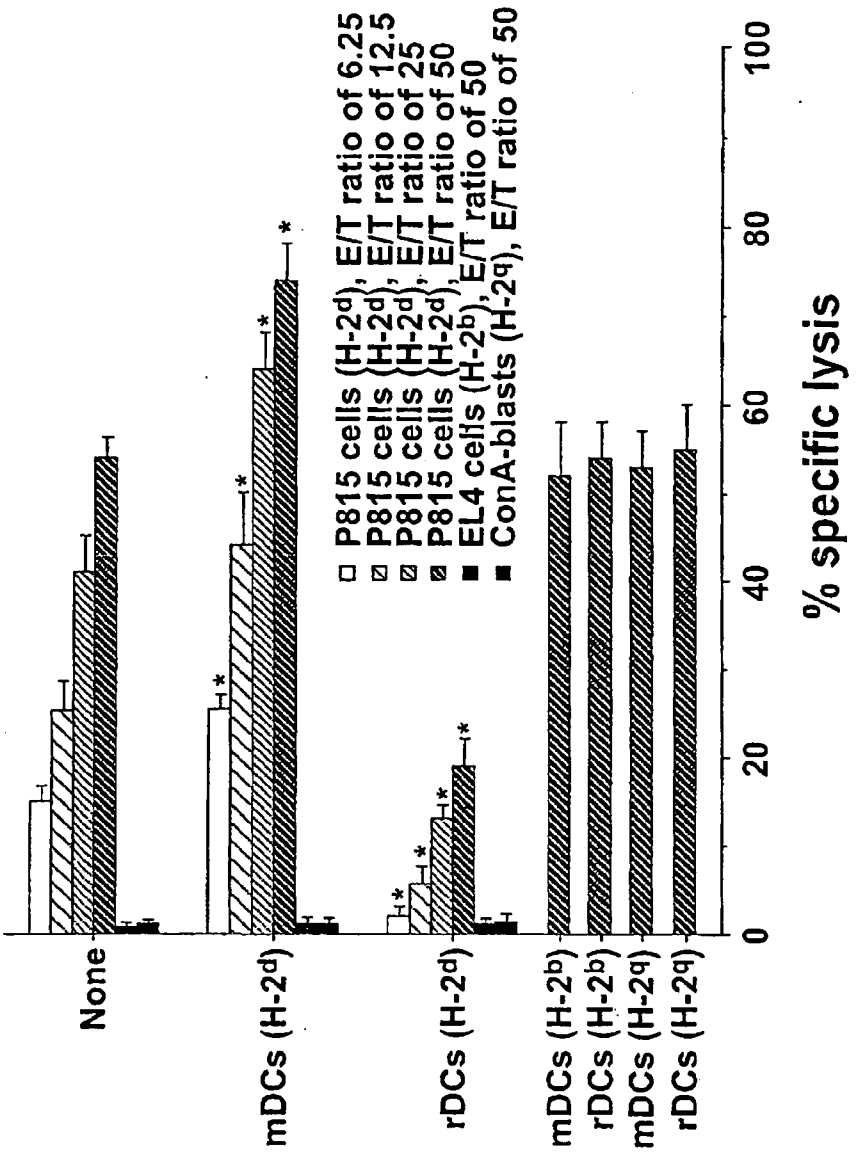

P815 cells (H-2d), E/T ratio of 6.25
P815 cells (H-2d), E/T ratio of 12.5
P815 cells (H-2d), E/T ratio of 25
P815 cells (H-2d), E/T ratio of 50
EL4 cells (H-2b), E/T ratio of 50
ConA-blasts (H-2q), E/T ratio of 50
% specific lysis
0
20
40
60
80
100
None
mDCs (H-2d)
rDCs (H-2d)
mDCs (H-2b)
rDCs (H-2b)
mDCs (H-2q)
rDCs (H-2q)

Fig. 7E

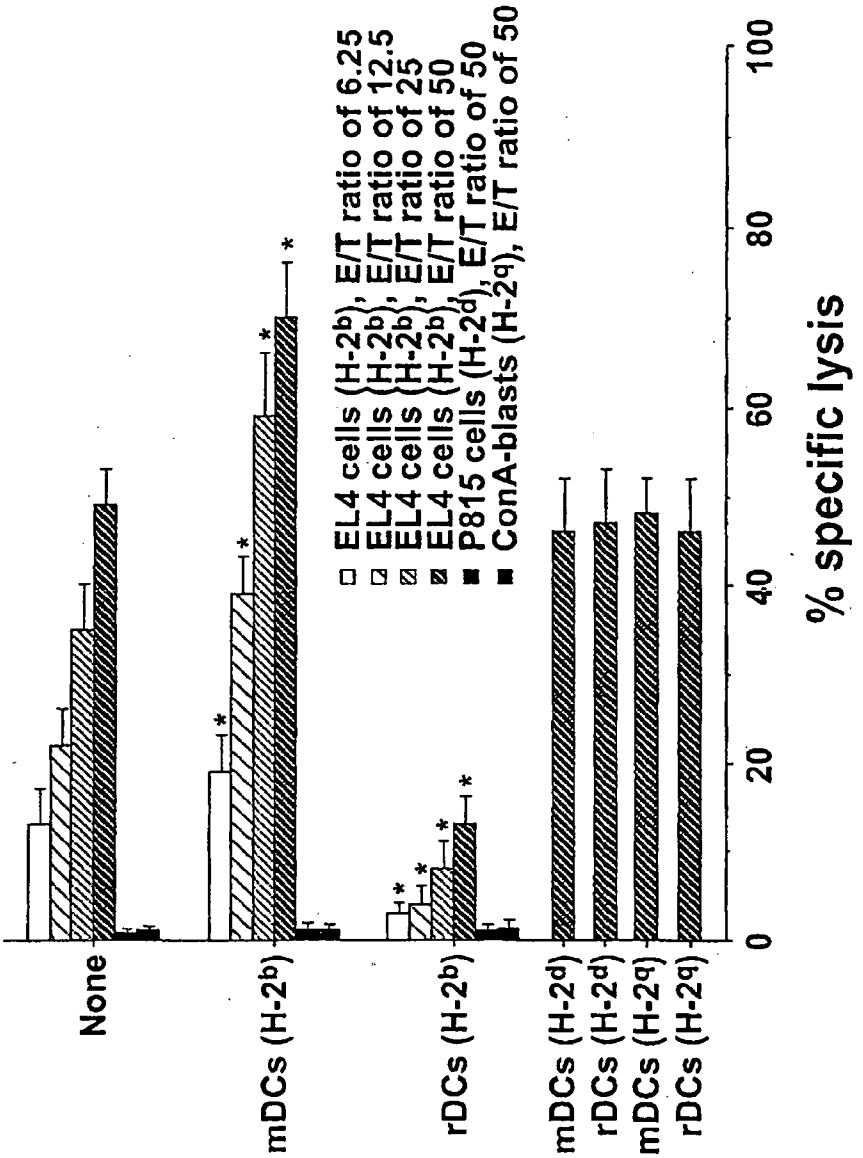

EL4 cells (H-2b), E/T ratio of 6.25
EL4 cells (H-2b), E/T ratio of 12.5
EL4 cells (H-2b), E/T ratio of 25
EL4 cells (H-2b), E/T ratio of 50
P815 cells (H-2d), E/T ratio of 50
ConA-blasts (H-2q), E/T ratio of 50
% specific lysis
0
20
40
60
80
100
None
mDCs (H-2b)
rDCs (H-2b)
mDCs (H-2d)
rDCs (H-2d)
mDCs (H-2q)
rDCs (H-2q)

Fig. 7F

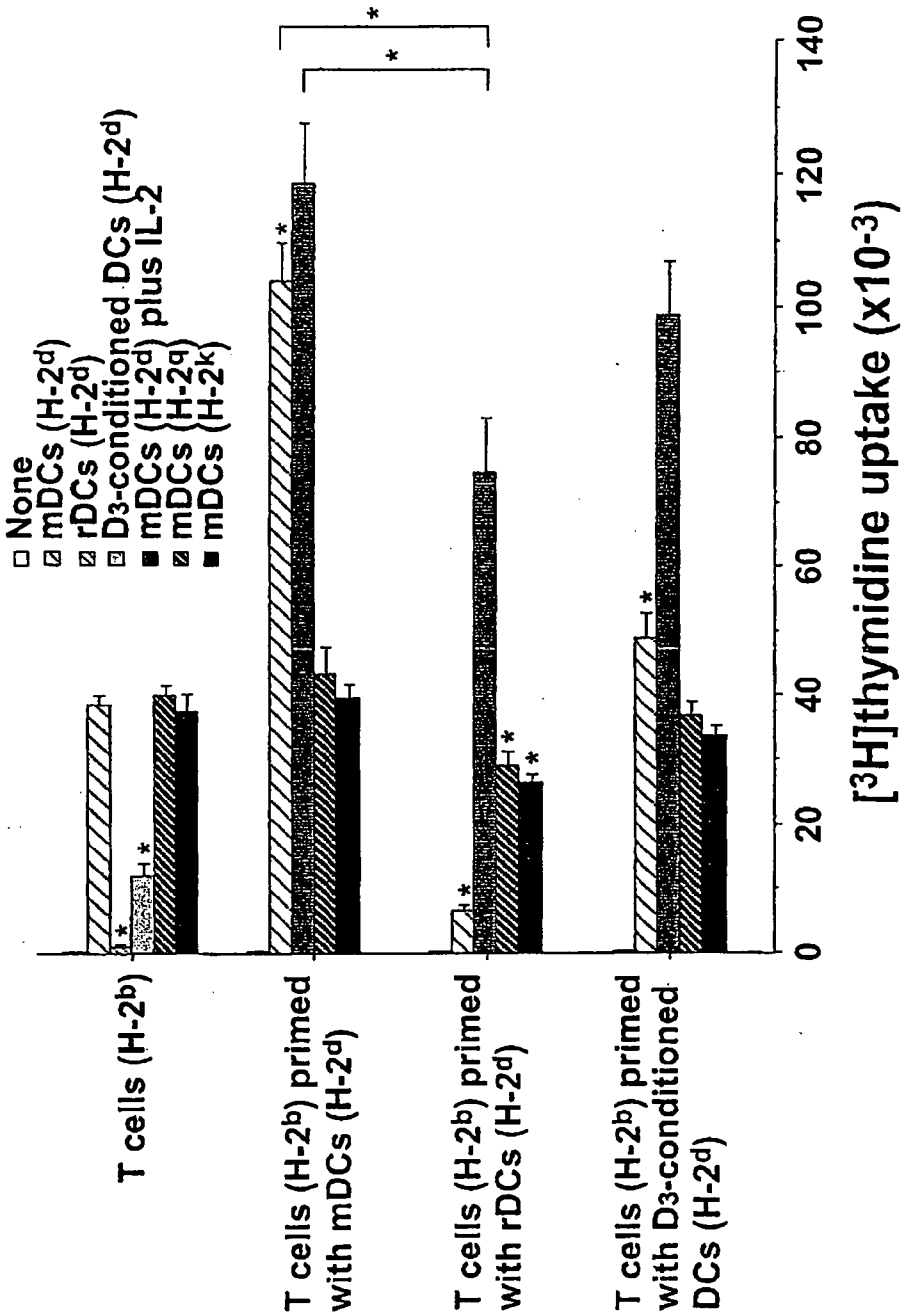

None
mDCs (H-2d)
rDCs (H-2d)
D3-conditioned DCs (H-2d)
mDCs (H-2d) plus IL-2
mDCs (H-2q)
mDCs (H-2k)
[3H]thymidine uptake (x10-3)
0
20
40
60
80
100
120
140
T cells (H-2b)
T cells (H-2b) primed with mDCs (H-2d)
T cells (H-2b) primed with rDCs (H-2d)
T cells (H-2b) primed with D3-conditioned DCs (H-2d)

Fig. 7G

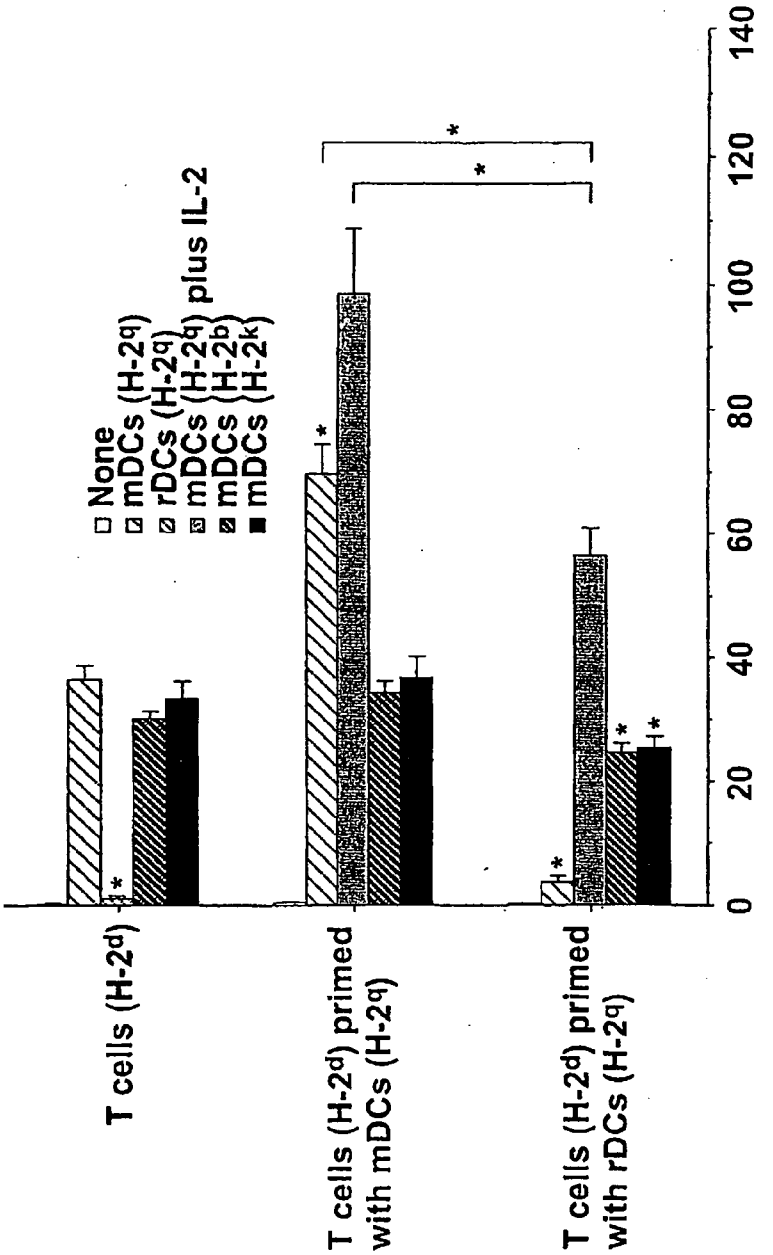

None
mDCs (H-2q)
rDCs (H-2q)
mDCs (H-2d) plus IL-2
mDCs (H-2b)
mDCs (H-2k)
[3H]thymidine uptake (x10-3)
0
20
40
60
80
100
120
140
T cells (H-2d)
T cells (H-2d) primed with mDCs (H-2q)
T cells (H-2d) primed with rDCs (H-2q)
*

Fig. 7H

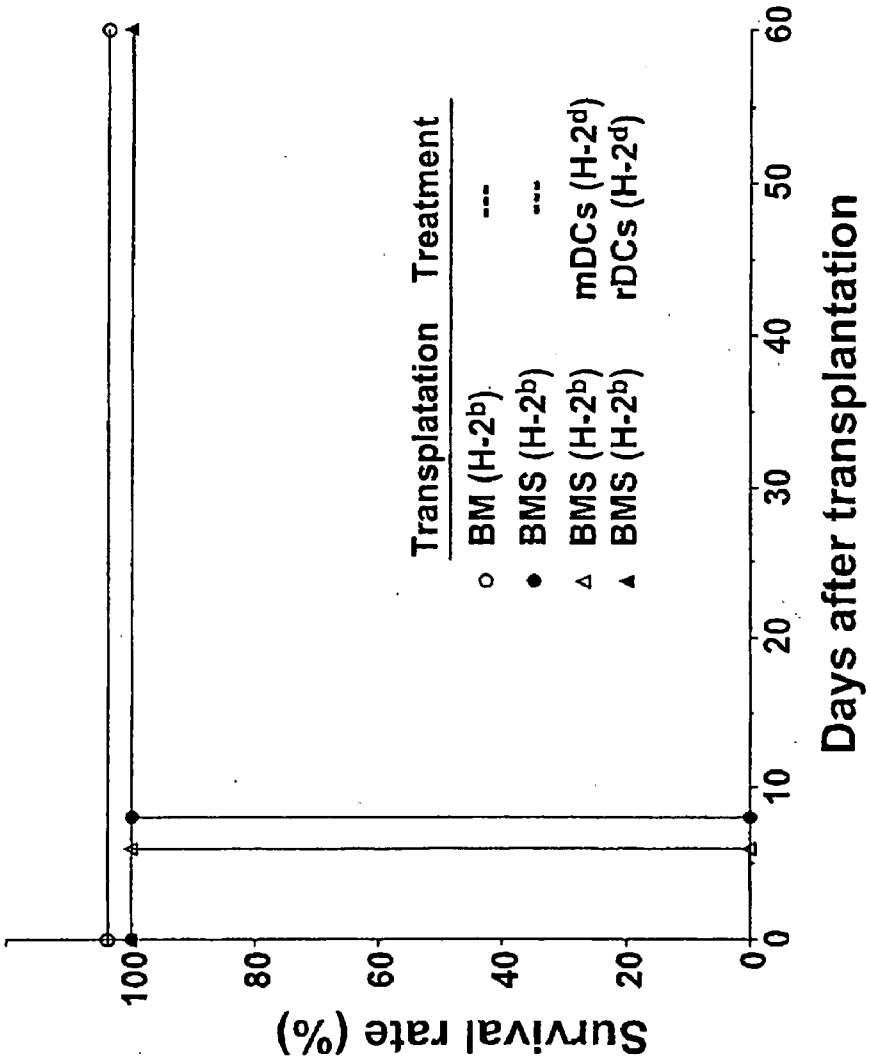

Survival rate (%)
100
80
60
40
20
0
0
10
20
30
40
50
60
Days after transplantation
Transplatation Treatment
BM (H-2b) ---
BMS (H-2b) ---
BMS (H-2b) mDCs (H-2d)
BMS (H-2b) rDCs (H-2d)

Fig. 8A

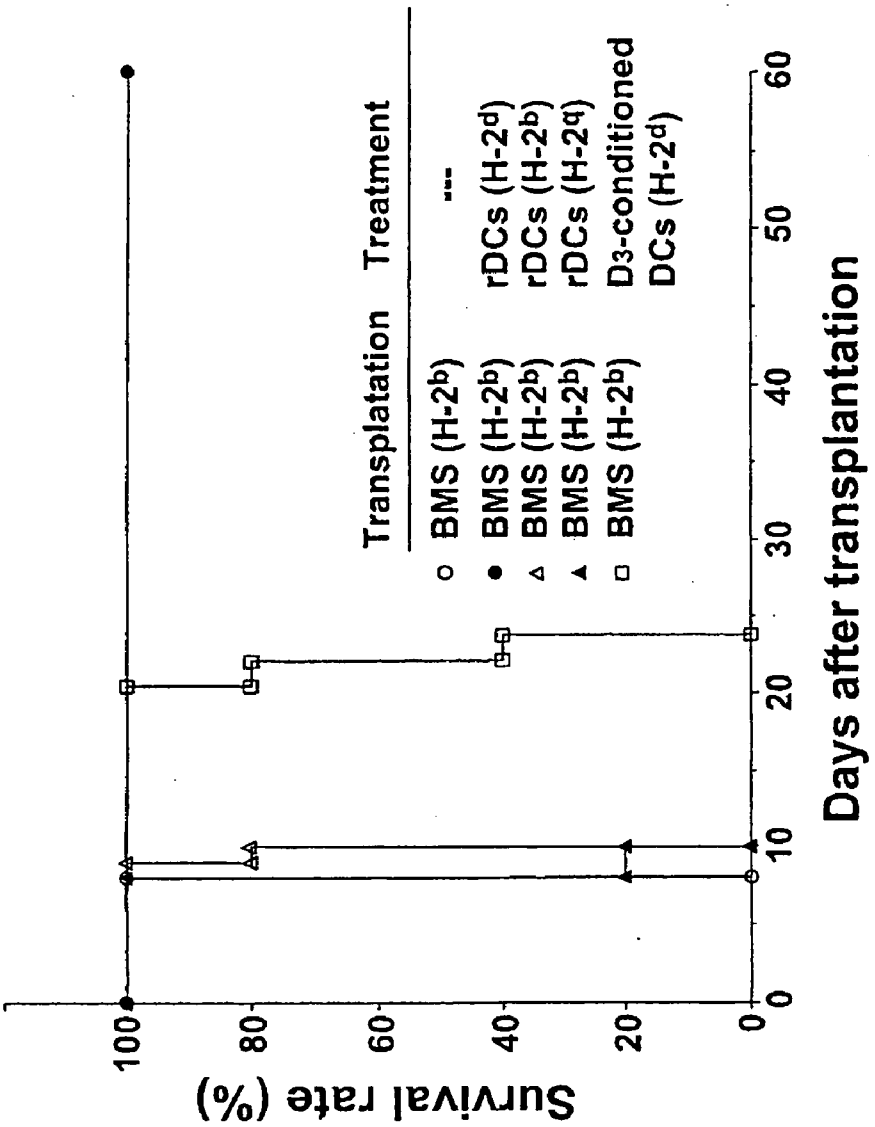
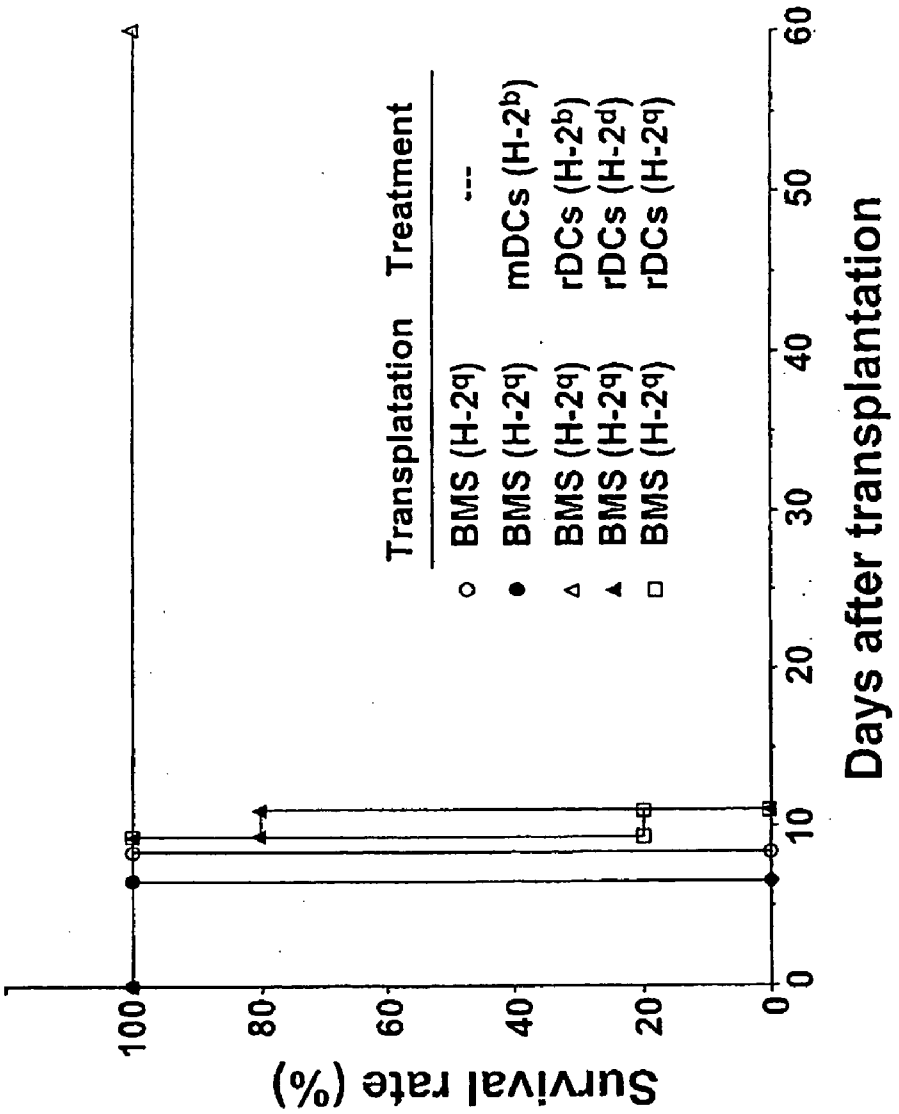

Survival rate (%)
100
80
60
40
20
0
0
10
20
30
40
50
60
Days after transplantation
Transplatation Treatment
BMS (H-2b) ---
BMS (H-2b) rDCs (H-2d)
BMS (H-2b) rDCs (H-2b)
BMS (H-2b) rDCs (H-2q)
BMS (H-2b) D3-conditioned DCs (H-2d)

Fig. 8B


Survival rate (%)
100
80
60
40
20
0
0
10
20
30
40
50
60
Days after transplantation
Transplatation
Treatment
BMS (H-2q)

BMS (H-2q)
mDCs (H-2b)
BMS (H-2q)
rDCs (H-2b)
BMS (H-2q)
rDCs (H-2d)
BMS (H-2q)
rDCs (H-2q)

Fig. 8C

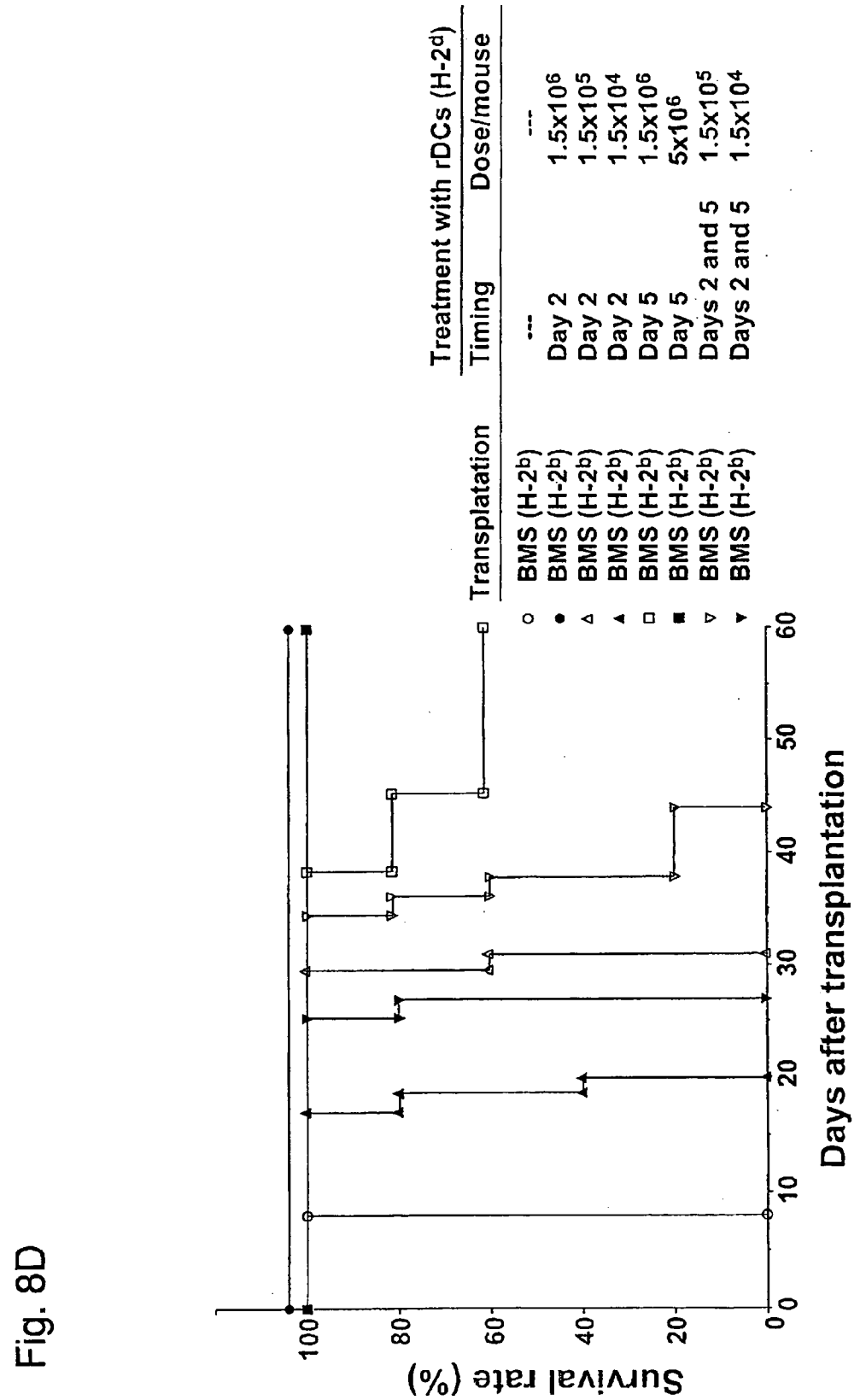

Survival rate (%)
100
80
60
40
20
0
0
10
20
30
40
50
60
Days after transplantation
Transplatation
Treatment with rDCs (H-2d)
Timing
Dose/mouse
BMS (H-2b) --- ---
BMS (H-2b) Day 2 1.5x10^6
BMS (H-2b) Day 2 1.5x10^5
BMS (H-2b) Day 2 1.5x10^4
BMS (H-2b) Day 5 1.5x10^6
BMS (H-2b) Day 5 5x10^6
BMS (H-2b) Days 2 and 5 1.5x10^5
BMS (H-2b) Days 2 and 5 1.5x10^4

Fig. 8D

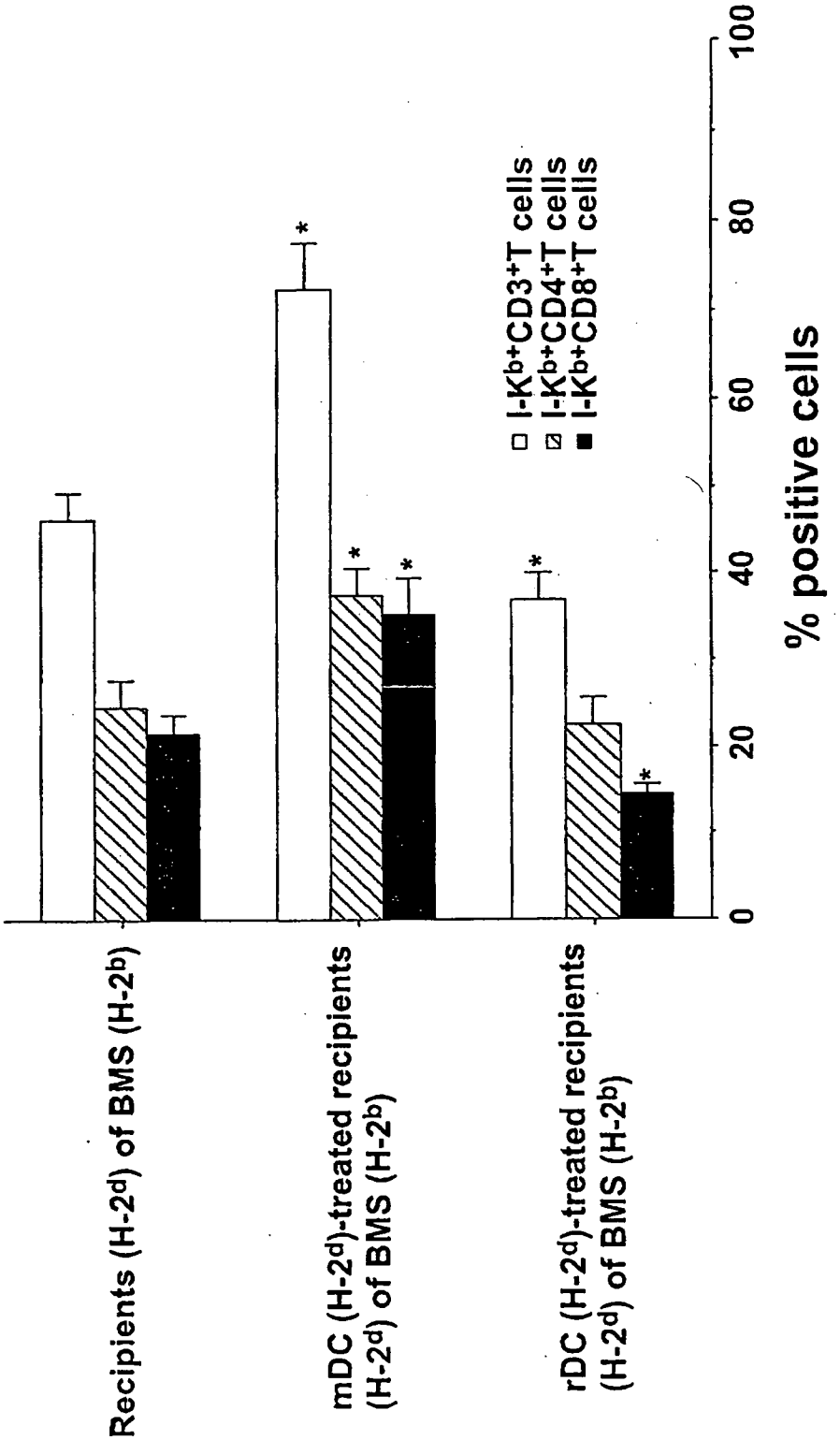

% positive cells
0
20
40
60
80
100
I-K^{b+}CD3^{+}T cells
I-K^{b+}CD4^{+}T cells
I-K^{b+}CD8^{+}T cells
*
Recipients (H-2^{d}) of BMS (H-2^{b})
mDC (H-2^{d})-treated recipients (H-2^{d}) of BMS (H-2^{b})
rDC (H-2^{d})-treated recipients (H-2^{d}) of BMS (H-2^{b})

Fig. 9A

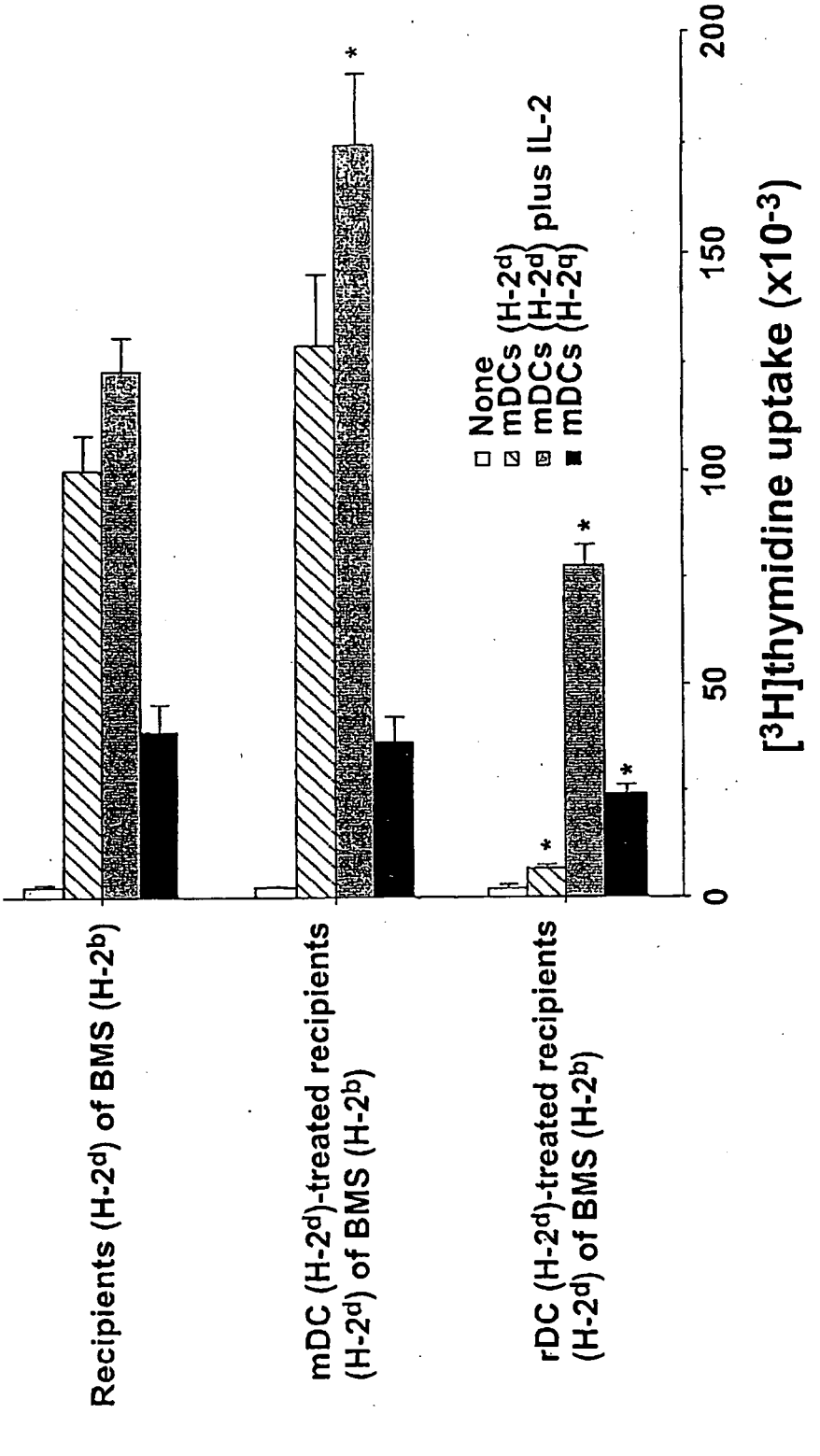

[3H]thymidine uptake (x10-3)
0
50
100
150
200
None
mDCs (H-2d)
mDCs (H-2d) plus IL-2
mDCs (H-2q)
*
Recipients (H-2d) of BMS (H-2b)
mDC (H-2d)-treated recipients (H-2d) of BMS (H-2b)
rDC (H-2d)-treated recipients (H-2d) of BMS (H-2b)

Fig. 9B

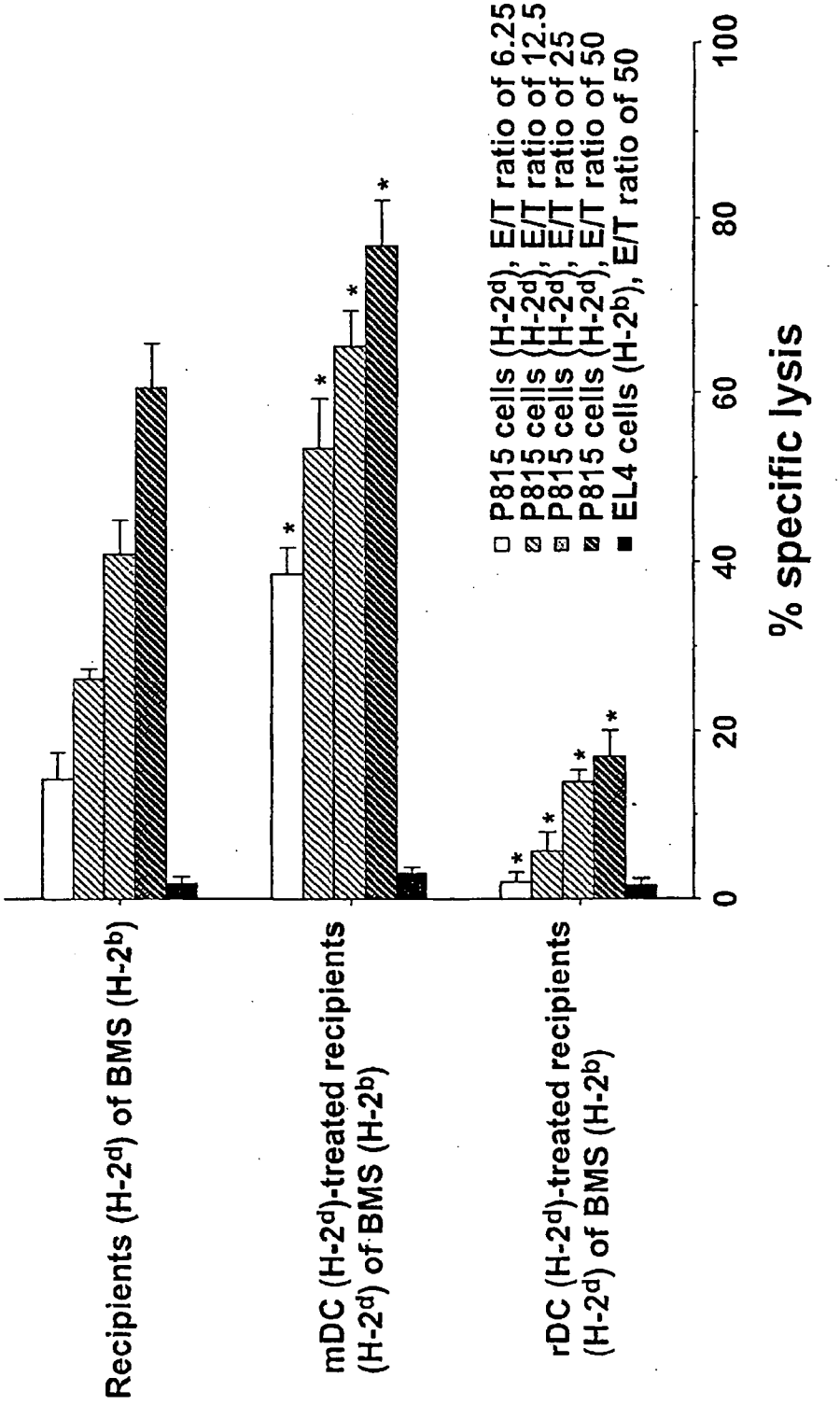

P815 cells (H-2d), E/T ratio of 6.25
P815 cells (H-2d), E/T ratio of 12.5
P815 cells (H-2d), E/T ratio of 25
P815 cells (H-2d), E/T ratio of 50
EL4 cells (H-2b), E/T ratio of 50
% specific lysis
0
20
40
60
80
100
Recipients (H-2d) of BMS (H-2b)
mDC (H-2d)-treated recipients (H-2d) of BMS (H-2b)
rDC (H-2d)-treated recipients (H-2d) of BMS (H-2b)

Fig. 9C

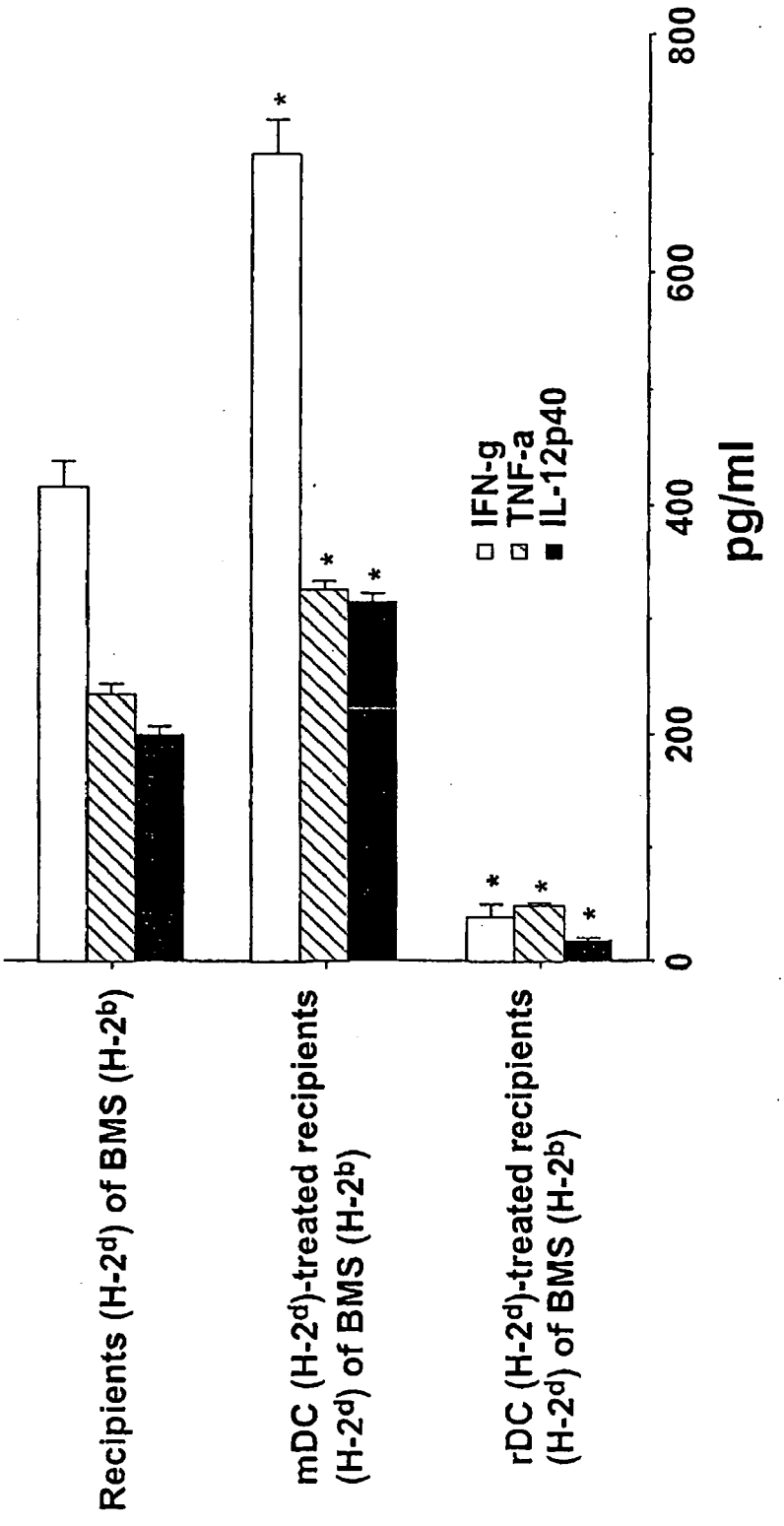

800
600
400
200
0
pg/ml
IFN-g
TNF-a
IL-12p40
*
Recipients (H-2d) of BMS (H-2b)
mDC (H-2d)-treated recipients (H-2d) of BMS (H-2b)
rDC (H-2d)-treated recipients (H-2d) of BMS (H-2b)

Fig. 9D

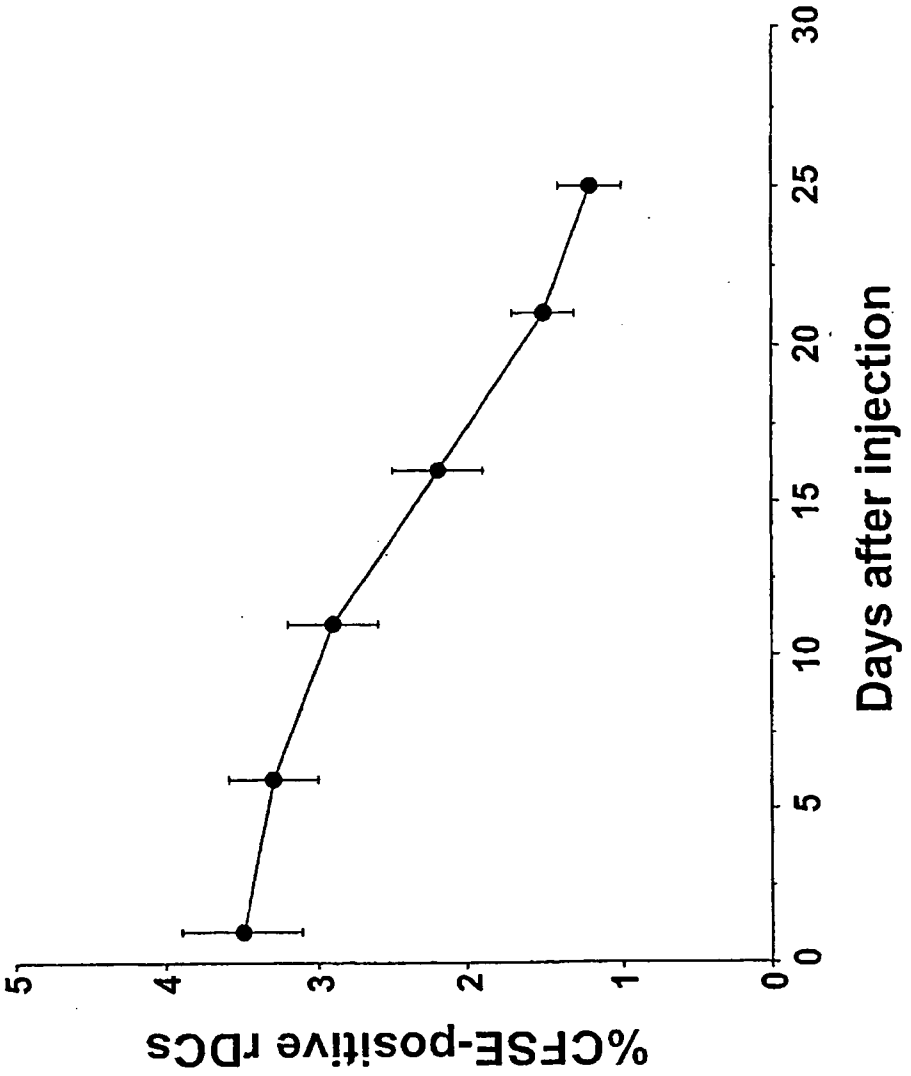

%CFSE-positive rDCs
0
1
2
3
4
5
0
5
10
15
20
25
30
Days after injection

Fig. 9E

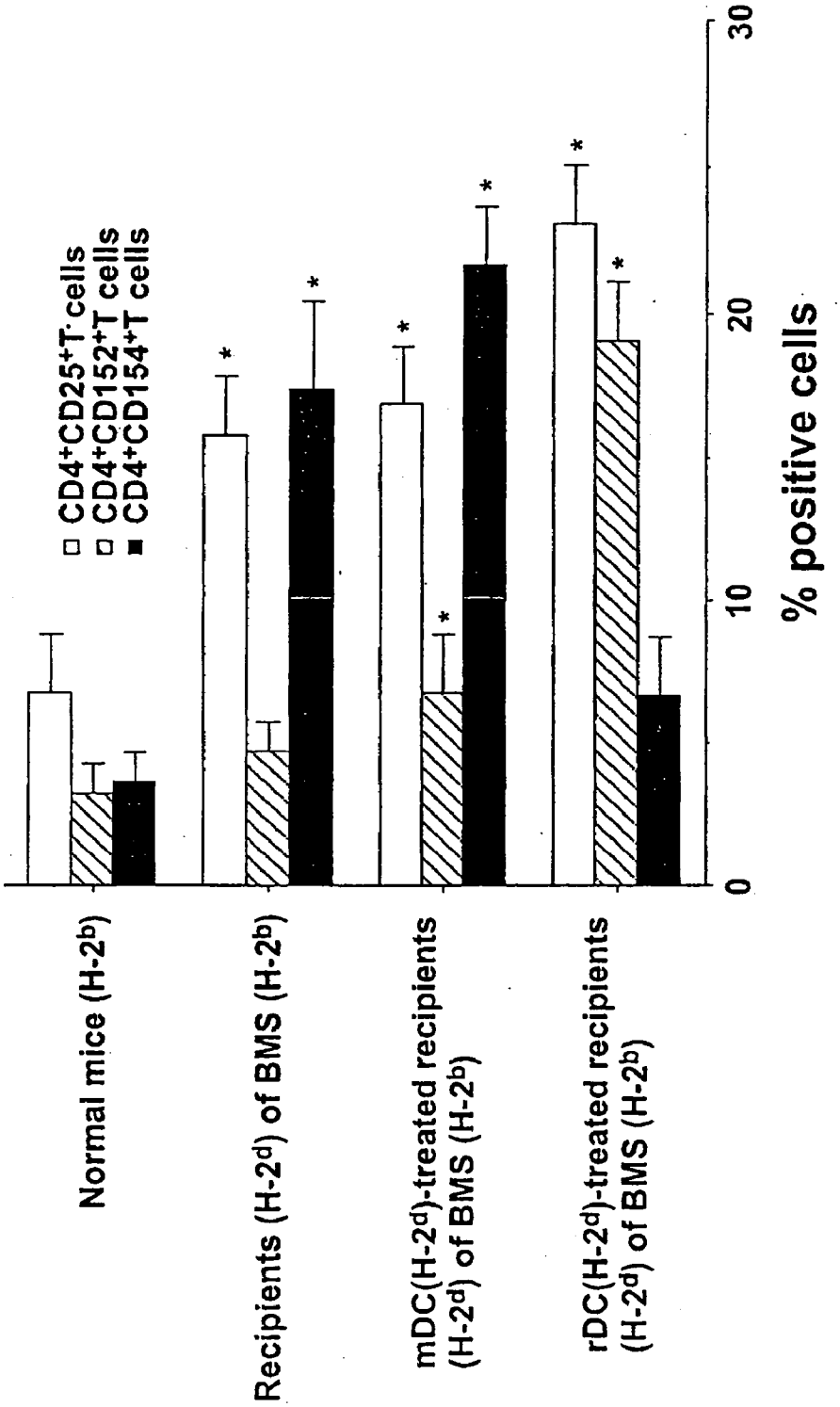

CD4+CD25+T cells
CD4+CD152+T cells
CD4+CD154+T cells
% positive cells
0
10
20
30
Normal mice (H-2b)
Recipients (H-2d) of BMS (H-2b)
mDC(H-2d)-treated recipients (H-2d) of BMS (H-2b)
rDC(H-2d)-treated recipients (H-2d) of BMS (H-2b)

Fig. 10A

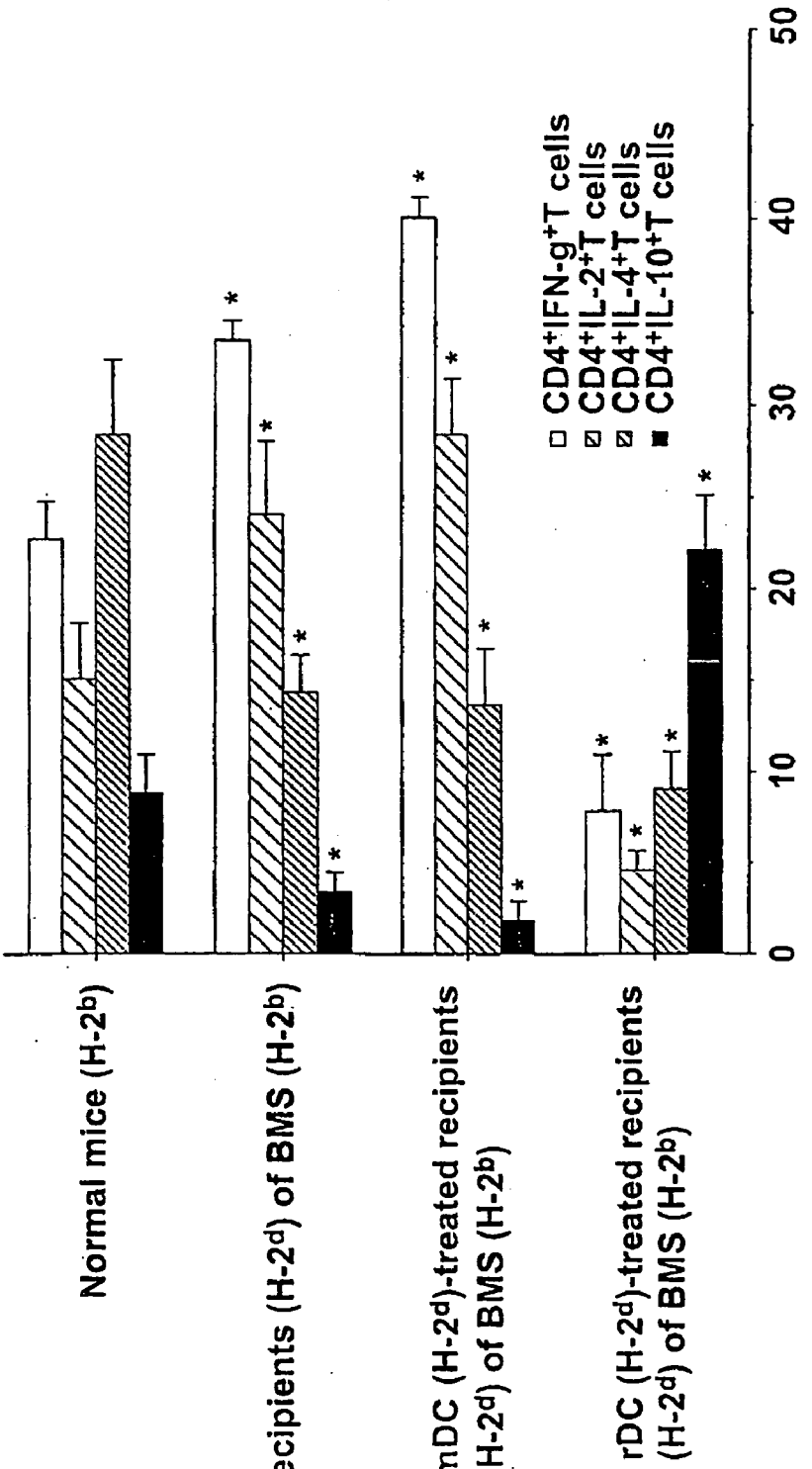

Normal mice (H-2b)
Recipients (H-2d) of BMS (H-2b)
mDC (H-2d)-treated recipients (H-2d) of BMS (H-2b)
rDC (H-2d)-treated recipients (H-2d) of BMS (H-2b)
CD4+IFN-g+T cells
CD4+IL-2+T cells
CD4+IL-4+T cells
CD4+IL-10+T cells
% positive cells
0
10
20
30
40
50
*

Fig. 10B

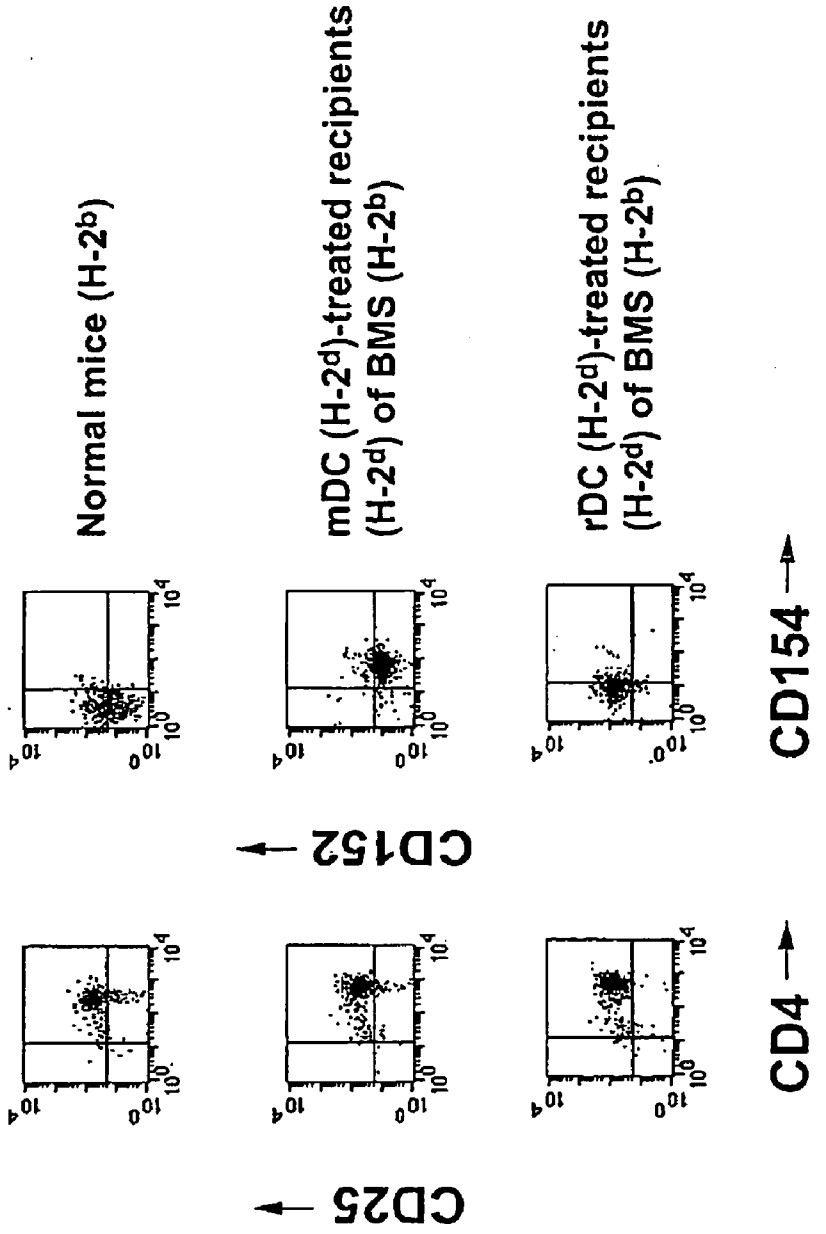

Normal mice (H-2b)
mDC (H-2d)-treated recipients (H-2d) of BMS (H-2b)
rDC (H-2d)-treated recipients (H-2d) of BMS (H-2b)
CD154
CD152
CD4
CD25

Fig. 10C

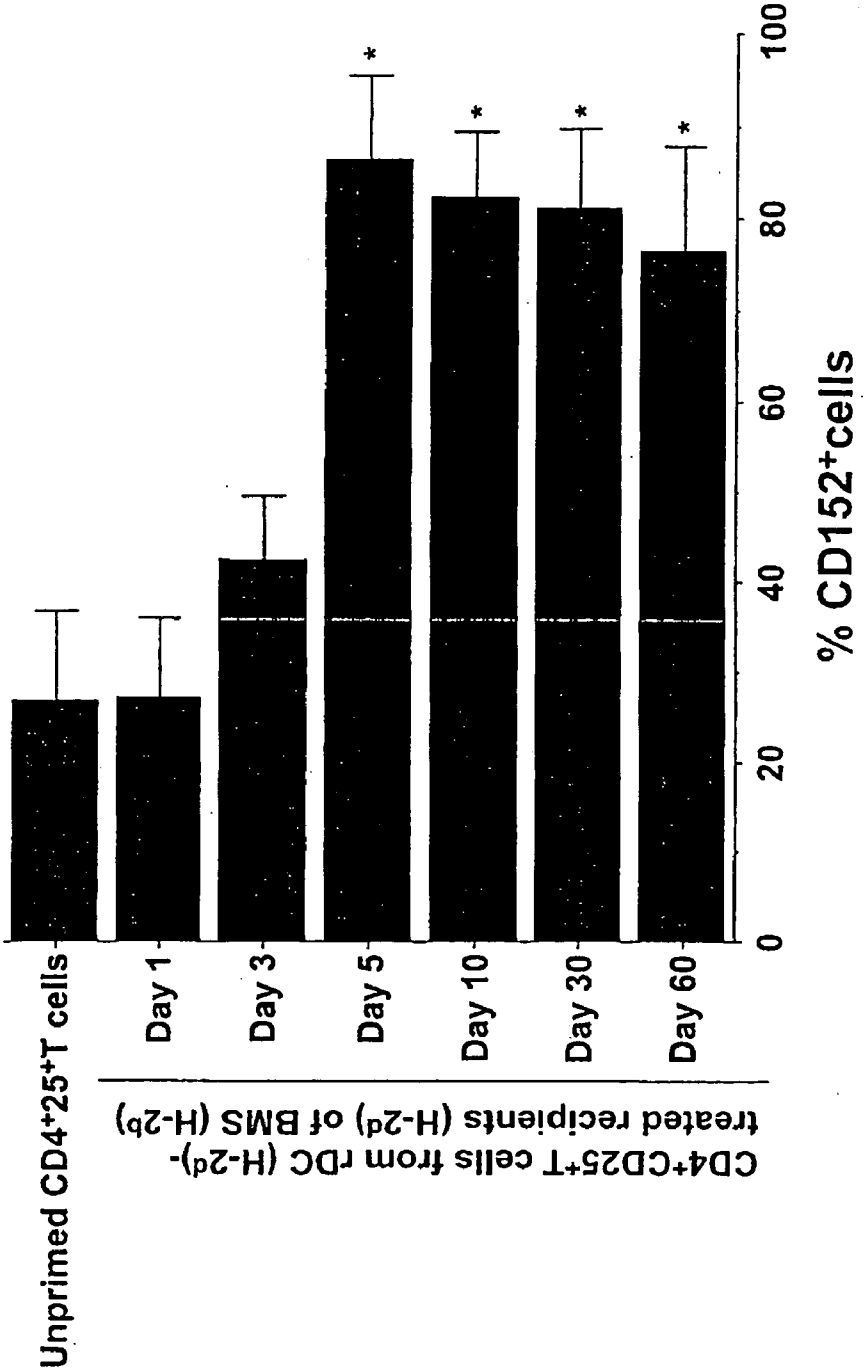

% CD152+cells
0
20
40
60
80
100
*
*
*
*
Unprimed CD4+25+T cells
Day 1
Day 3
Day 5
Day 10
Day 30
Day 60
CD4+CD25+T cells from rDC (H-2d)-
treated recipients (H-2d) of BMS (H-2b)

Fig. 10D

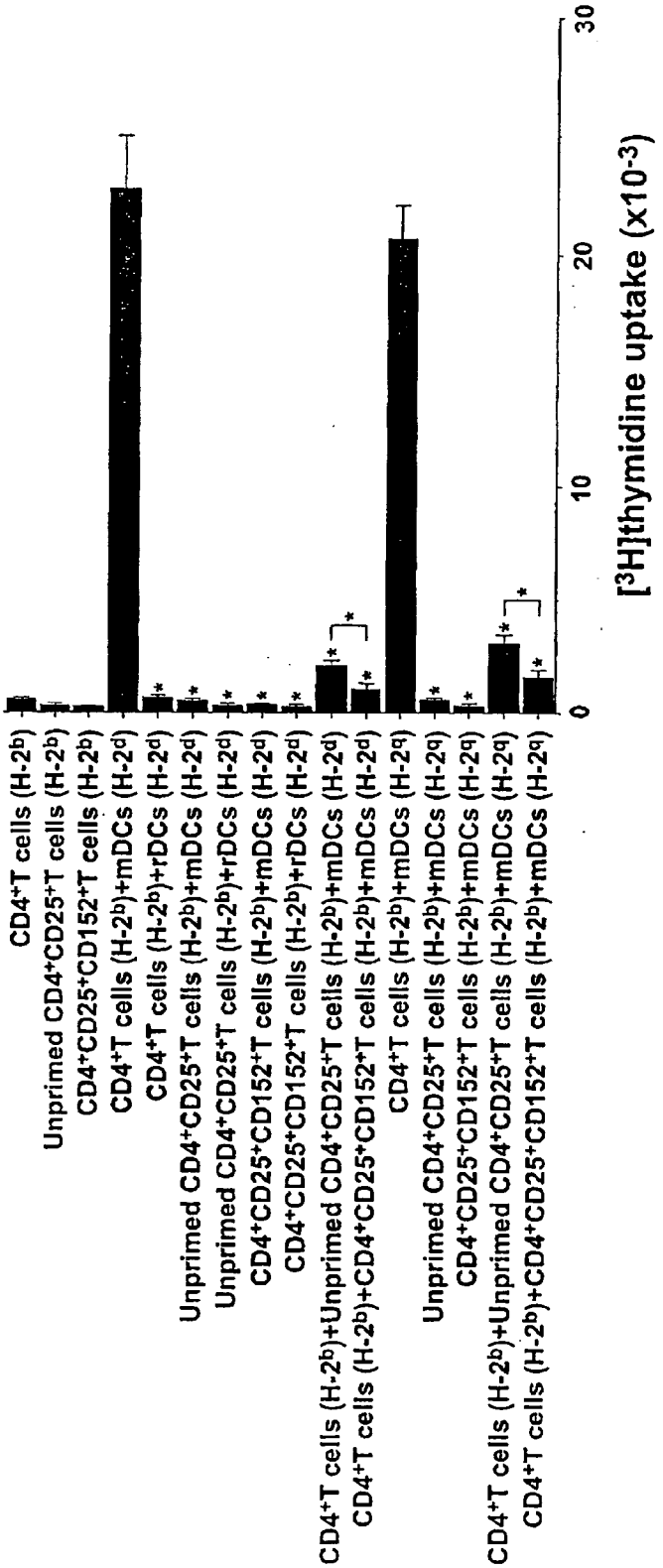

CD4+T cells (H-2b)
Unprimed CD4+CD25+T cells (H-2b)
CD4+CD25+CD152+T cells (H-2b)
CD4+T cells (H-2b)+mDCs (H-2d)
CD4+T cells (H-2b)+rDCs (H-2d)
Unprimed CD4+CD25+T cells (H-2b)+mDCs (H-2d)
Unprimed CD4+CD25+T cells (H-2b)+rDCs (H-2d)
CD4+CD25+CD152+T cells (H-2b)+mDCs (H-2d)
CD4+CD25+CD152+T cells (H-2b)+rDCs (H-2d)
CD4+T cells (H-2b)+Unprimed CD4+CD25+T cells (H-2b)+mDCs (H-2d)
CD4+T cells (H-2b)+CD4+CD25+CD152+T cells (H-2b)+mDCs (H-2d)
CD4+T cells (H-2b)+mDCs (H-2q)
Unprimed CD4+CD25+T cells (H-2b)+mDCs (H-2q)
CD4+CD25+CD152+T cells (H-2b)+mDCs (H-2q)
CD4+T cells (H-2b)+Unprimed CD4+CD25+T cells (H-2b)+mDCs (H-2q)
CD4+T cells (H-2b)+CD4+CD25+CD152+T cells (H-2b)+mDCs (H-2q)
0
10
20
30
[3H]thymidine uptake (x10-3)

Fig. 10E

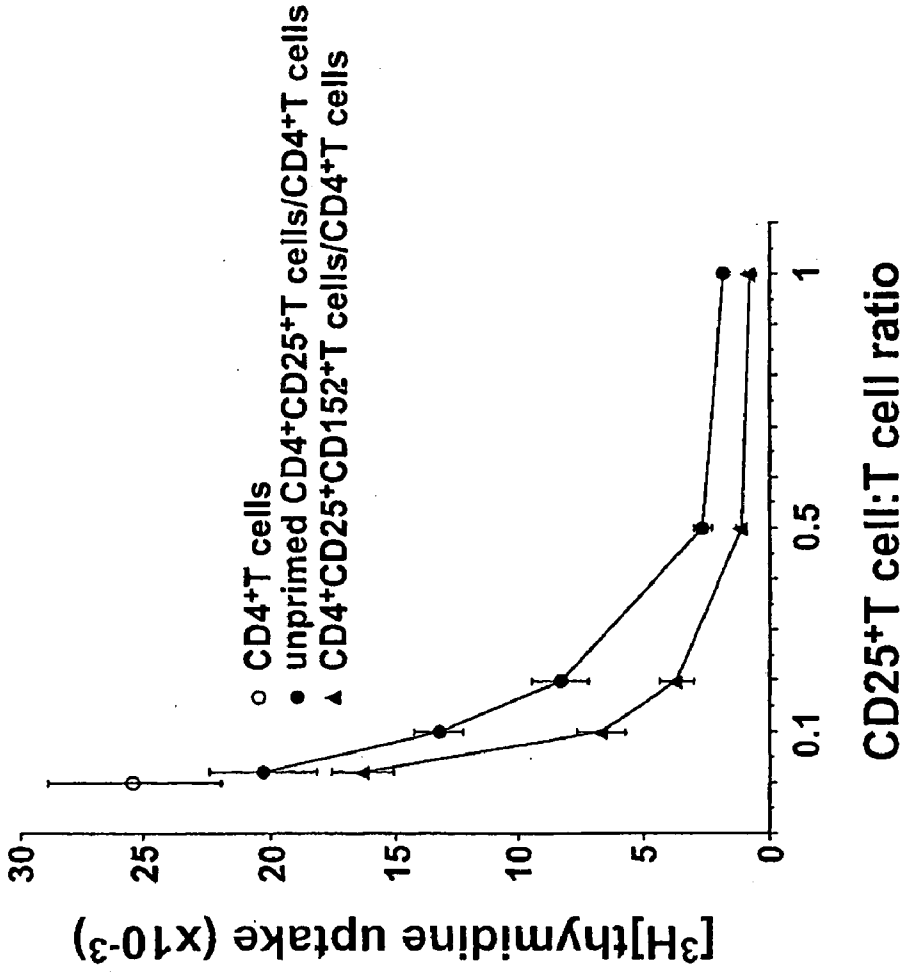

CD4+T cells
unprimed CD4+CD25+T cells/CD4+T cells
CD4+CD25+CD152+T cells/CD4+T cells
[3H]thymidine uptake (x10-3)
30
25
20
15
10
5
0
0.1
0.5
1
CD25+T cell:T cell ratio

Fig. 10F

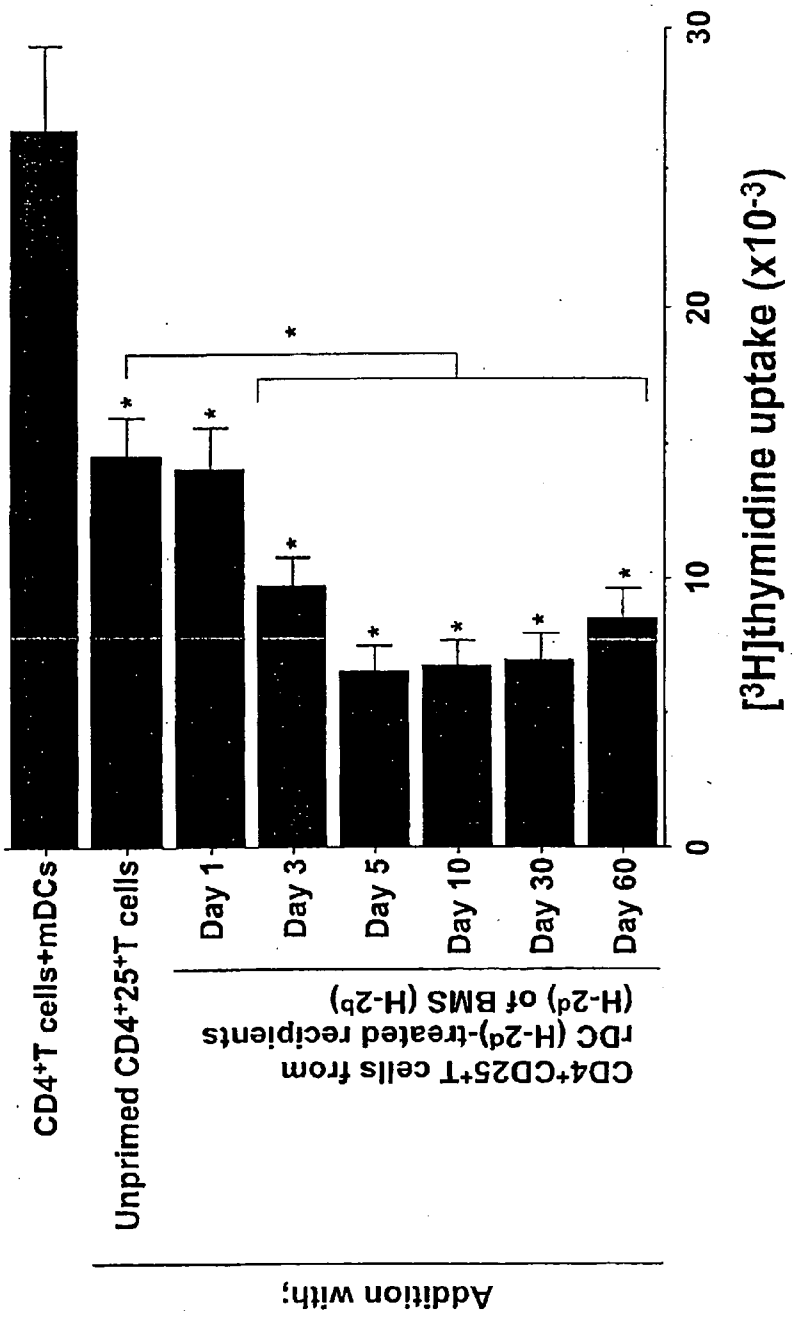

[3H]thymidine uptake (x10-3)
0
10
20
30
*
Addition with;
CD4+T cells+mDCs
Unprimed CD4+25+T cells
Day 1
Day 3
Day 5
Day 10
Day 30
Day 60
CD4+CD25+T cells from
rDC (H-2d)-treated recipients
(H-2d) of BMS (H-2b)

Fig. 10G

Fig. 10H

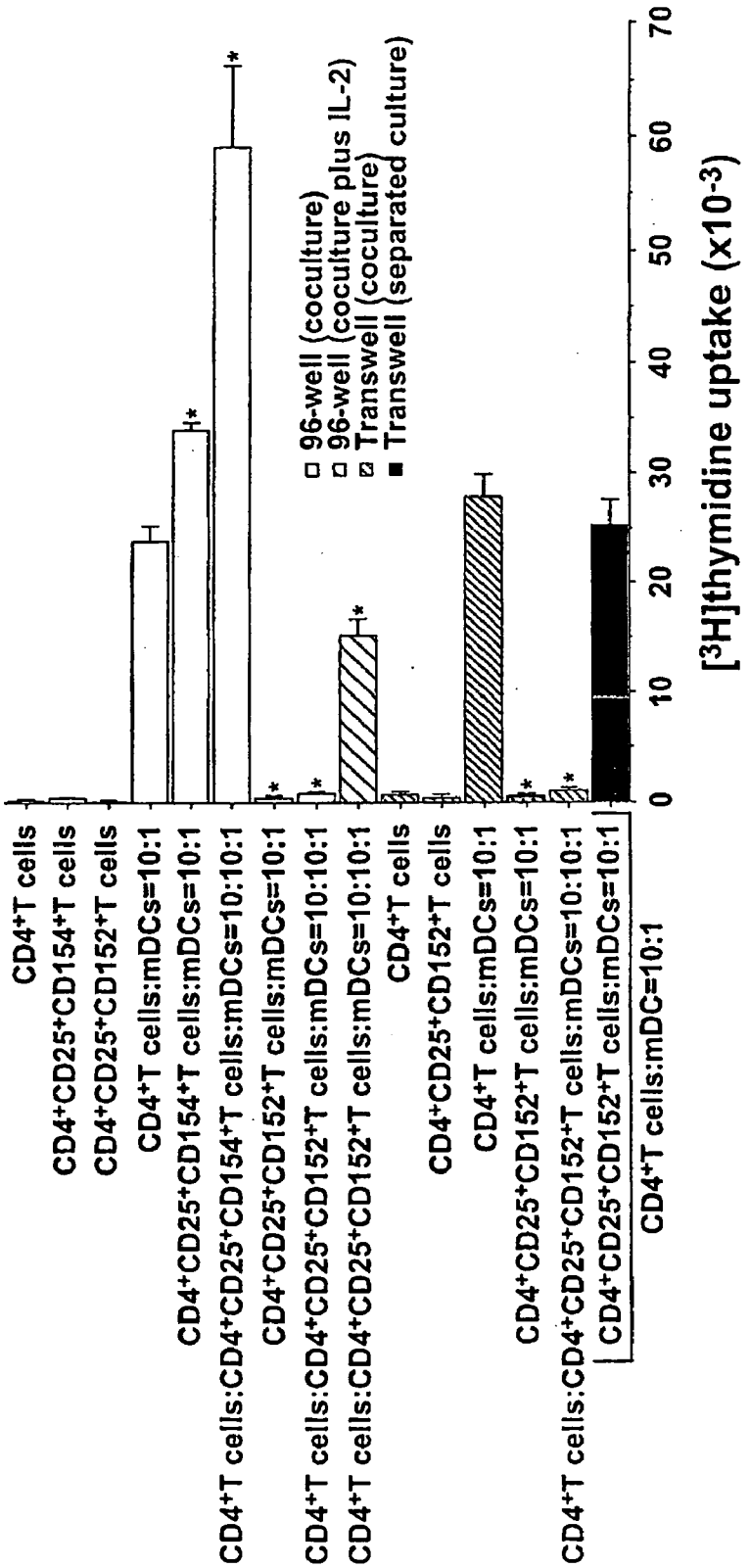
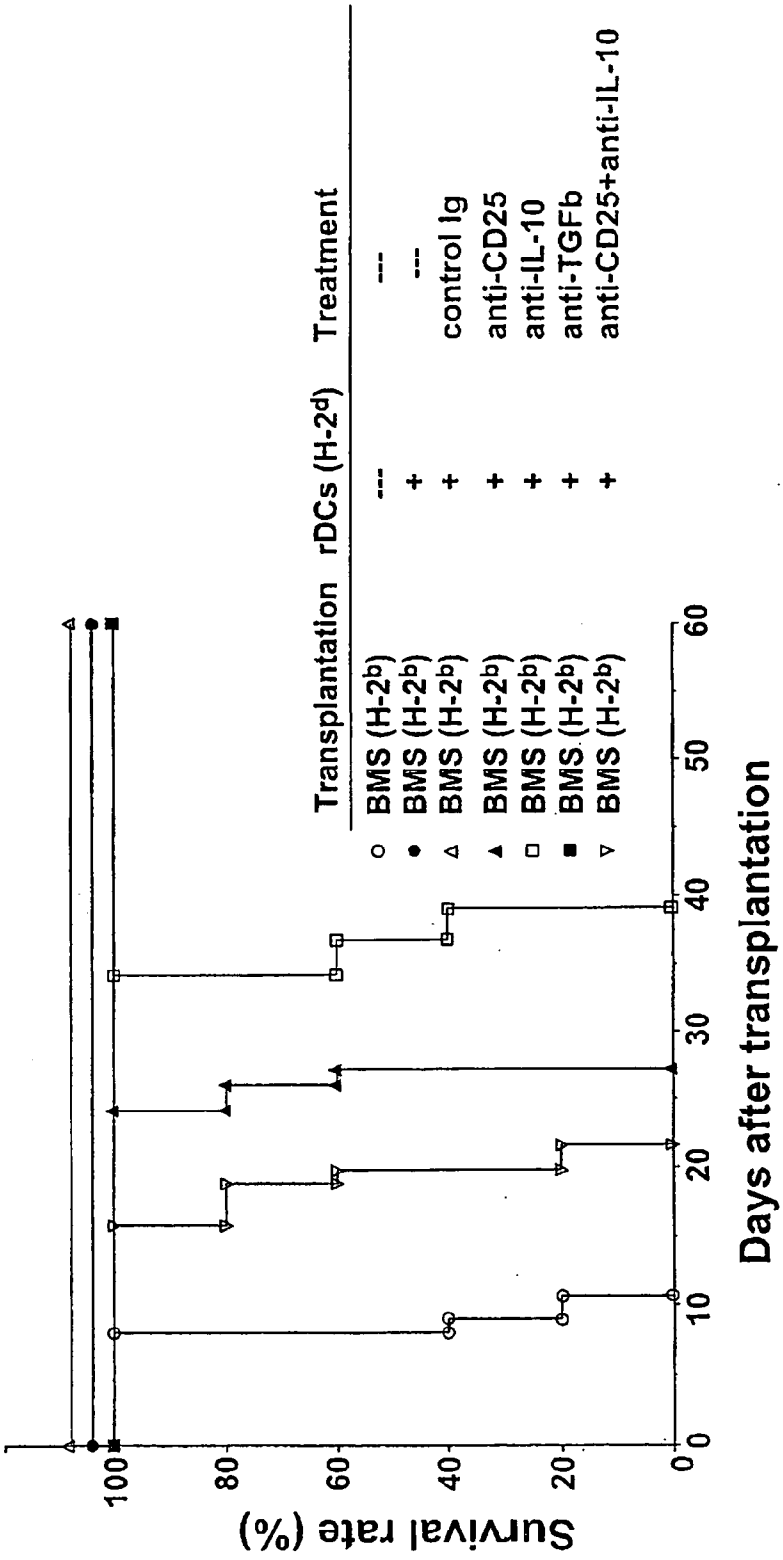

CD4+T cells
CD4+CD25+CD154+T cells
CD4+CD25+CD152+T cells
CD4+T cells:mDCs=10:1
CD4+CD25+CD154+T cells:mDCs=10:1
CD4+T cells:CD4+CD25+CD154+T cells:mDCs=10:10:1
CD4+CD25+CD152+T cells:mDCs=10:1
CD4+T cells:CD4+CD25+CD152+T cells:mDCs=10:10:1
CD4+T cells:CD4+CD25+CD152+T cells:mDCs=10:10:1
CD4+T cells
CD4+CD25+CD152+T cells
CD4+T cells:mDCs=10:1
CD4+CD25+CD152+T cells:mDCs=10:1
CD4+T cells:CD4+CD25+CD152+T cells:mDCs=10:10:1
CD4+T cells:CD4+CD25+CD152+T cells:mDCs=10:10:1
CD4+T cells:mDC=10:1
96-well (coculture)
96-well (coculture plus IL-2)
Transwell (coculture)
Transwell (separated culture)
0
10
20
30
40
50
60
70
[3H]thymidine uptake (x10-3)

Fig. 10I


Survival rate (%)
0
20
40
60
80
100
0
10
20
30
40
50
60
Days after transplantation
Transplantation
rDCs (H-2d)
Treatment
BMS (H-2b)

BMS (H-2b)
+

BMS (H-2b)
+
control Ig
BMS (H-2b)
+
anti-CD25
BMS (H-2b)
+
anti-IL-10
BMS (H-2b)
+
anti-TGFb
BMS (H-2b)
+
anti-CD25+anti-IL-10

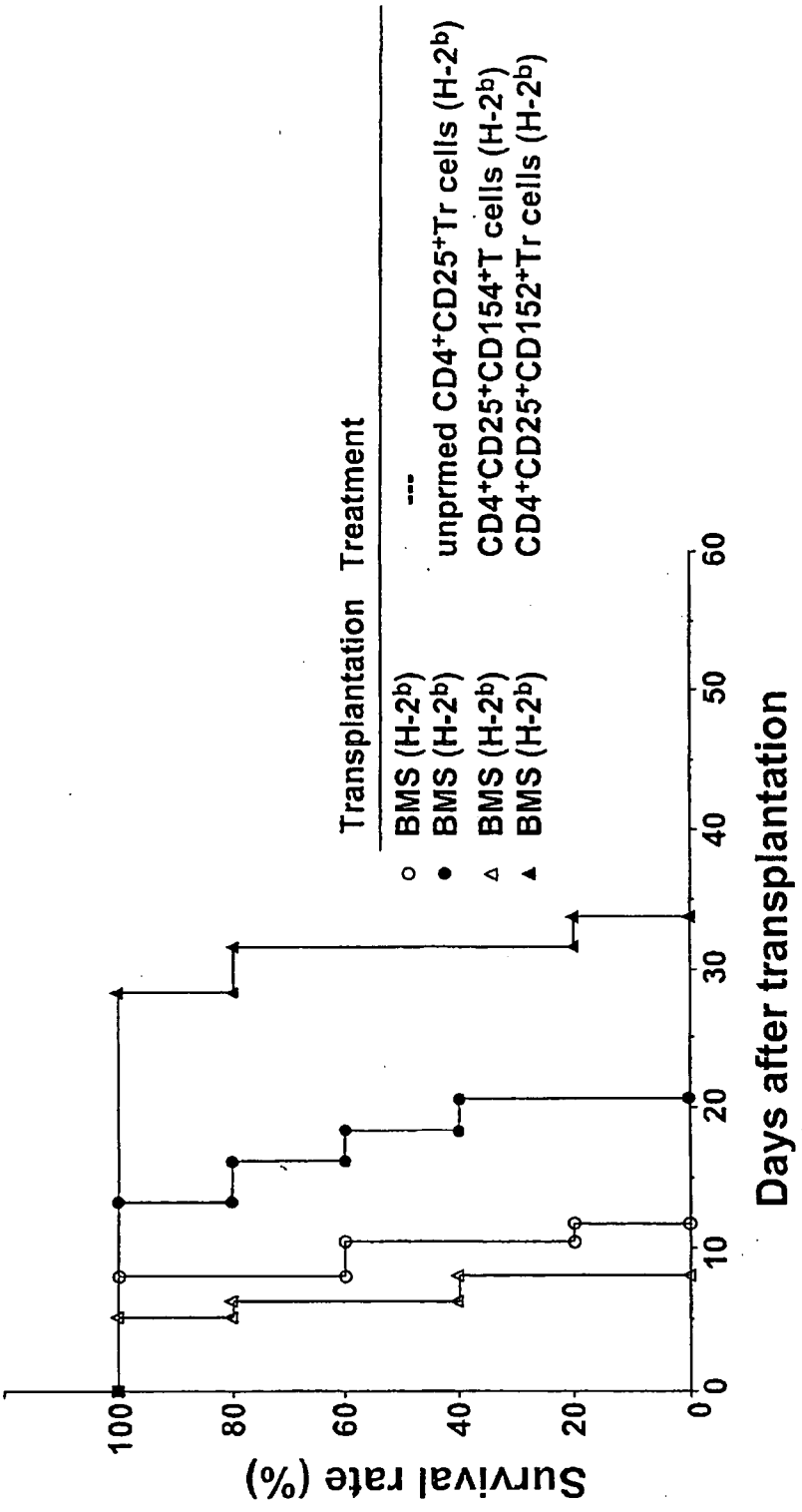

Survival rate (%)
100
80
60
40
20
0
0
10
20
30
40
50
60
Days after transplantation
Transplantation Treatment
BMS (H-2b) ---
BMS (H-2b) unprmed CD4+CD25+Tr cells (H-2b)
BMS (H-2b) CD4+CD25+CD154+T cells (H-2b)
BMS (H-2b) CD4+CD25+CD152+Tr cells (H-2b)

Fig. 10J

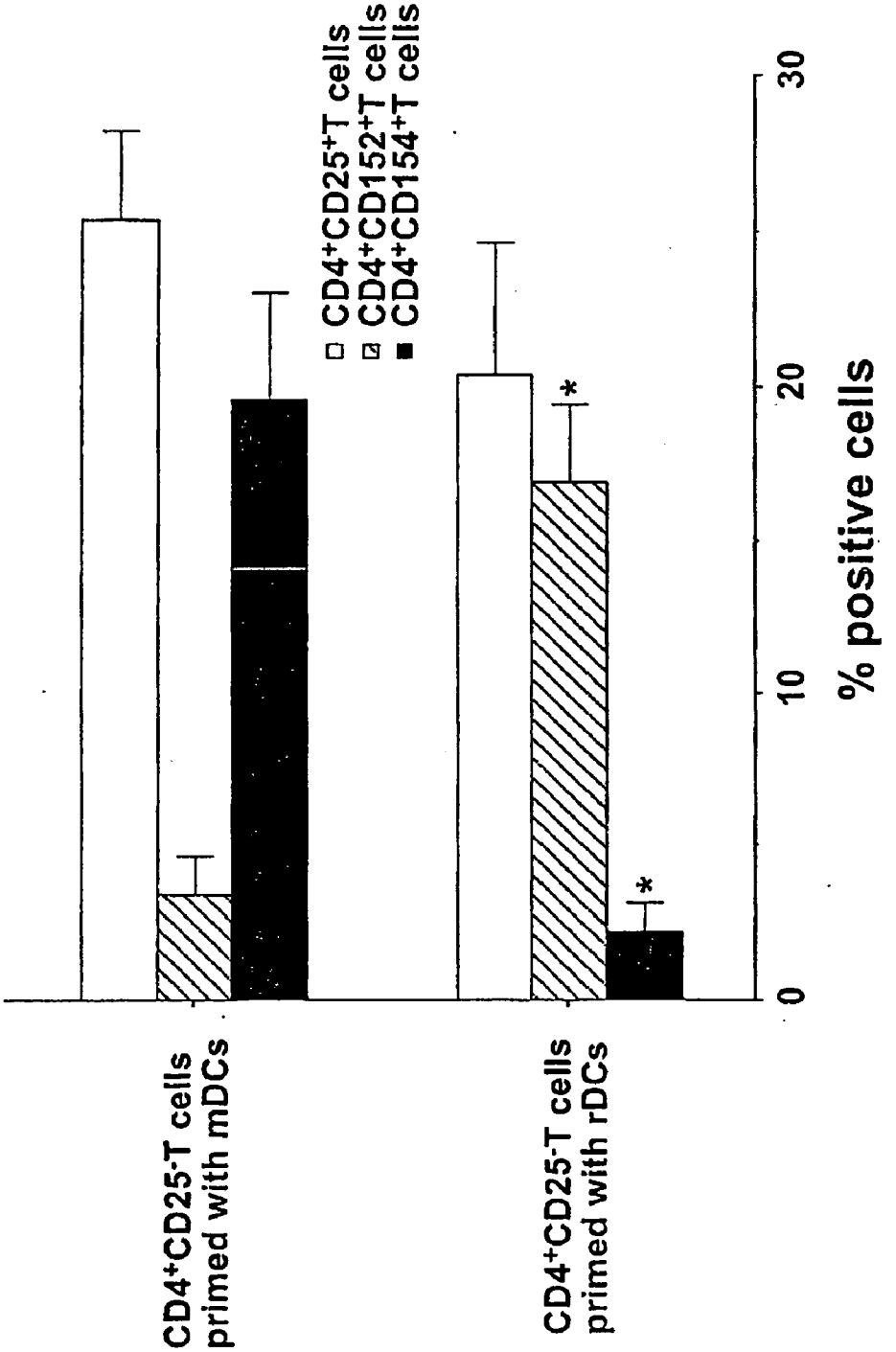

CD4+CD25+T cells
CD4+CD152+T cells
CD4+CD154+T cells
% positive cells
0
10
20
30
*
*
CD4+CD25-T cells primed with mDCs
CD4+CD25-T cells primed with rDCs

Fig. 11

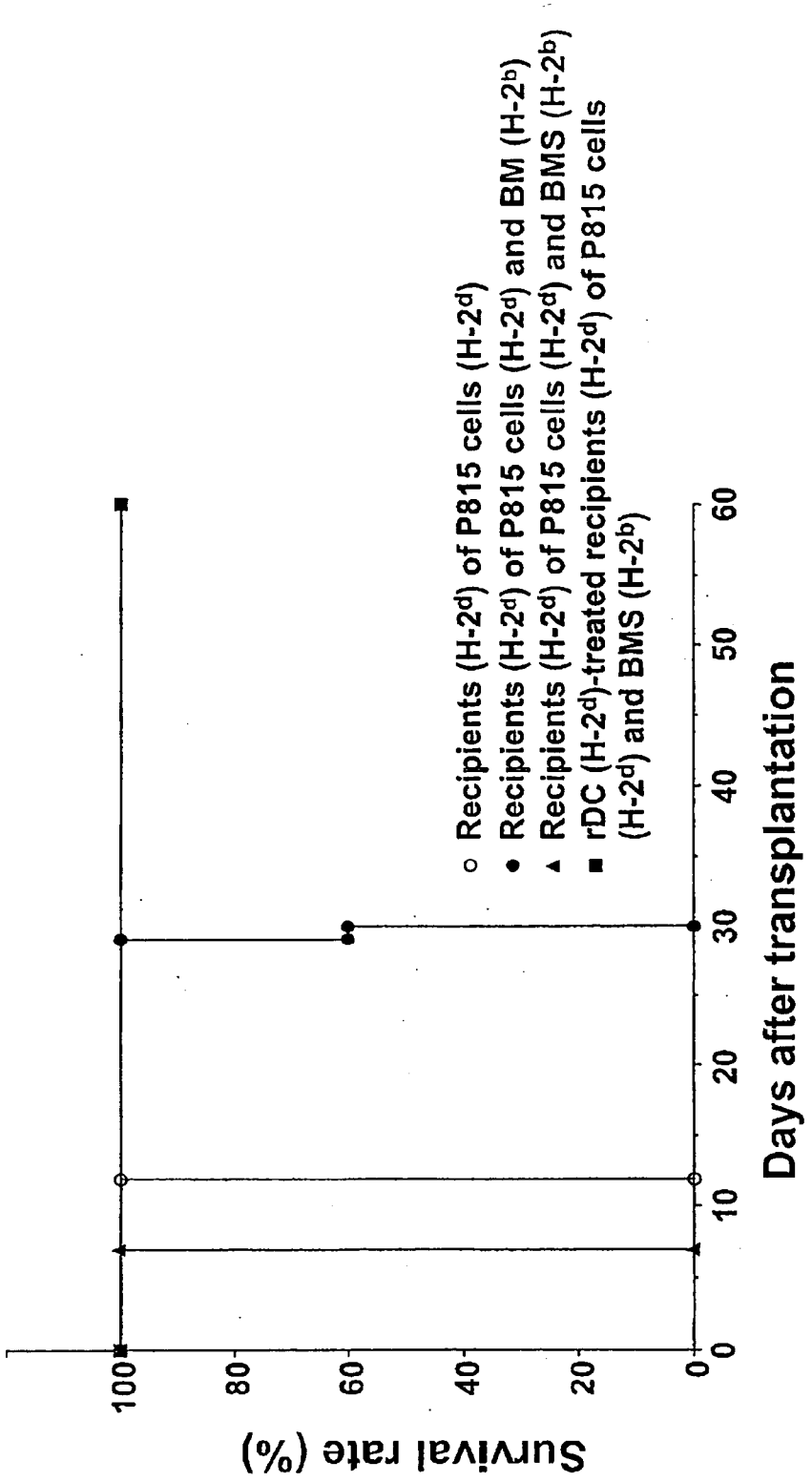

Survival rate (%)
100
80
60
40
20
0
0
10
20
30
40
50
60
Days after transplantation
○ Recipients (H-2d) of P815 cells (H-2d)
● Recipients (H-2d) of P815 cells (H-2d) and BM (H-2b)
▲ Recipients (H-2d) of P815 cells (H-2d) and BMS (H-2b)
■ rDC (H-2d)-treated recipients (H-2d) of P815 cells (H-2d) and BMS (H-2b)

Fig. 12A

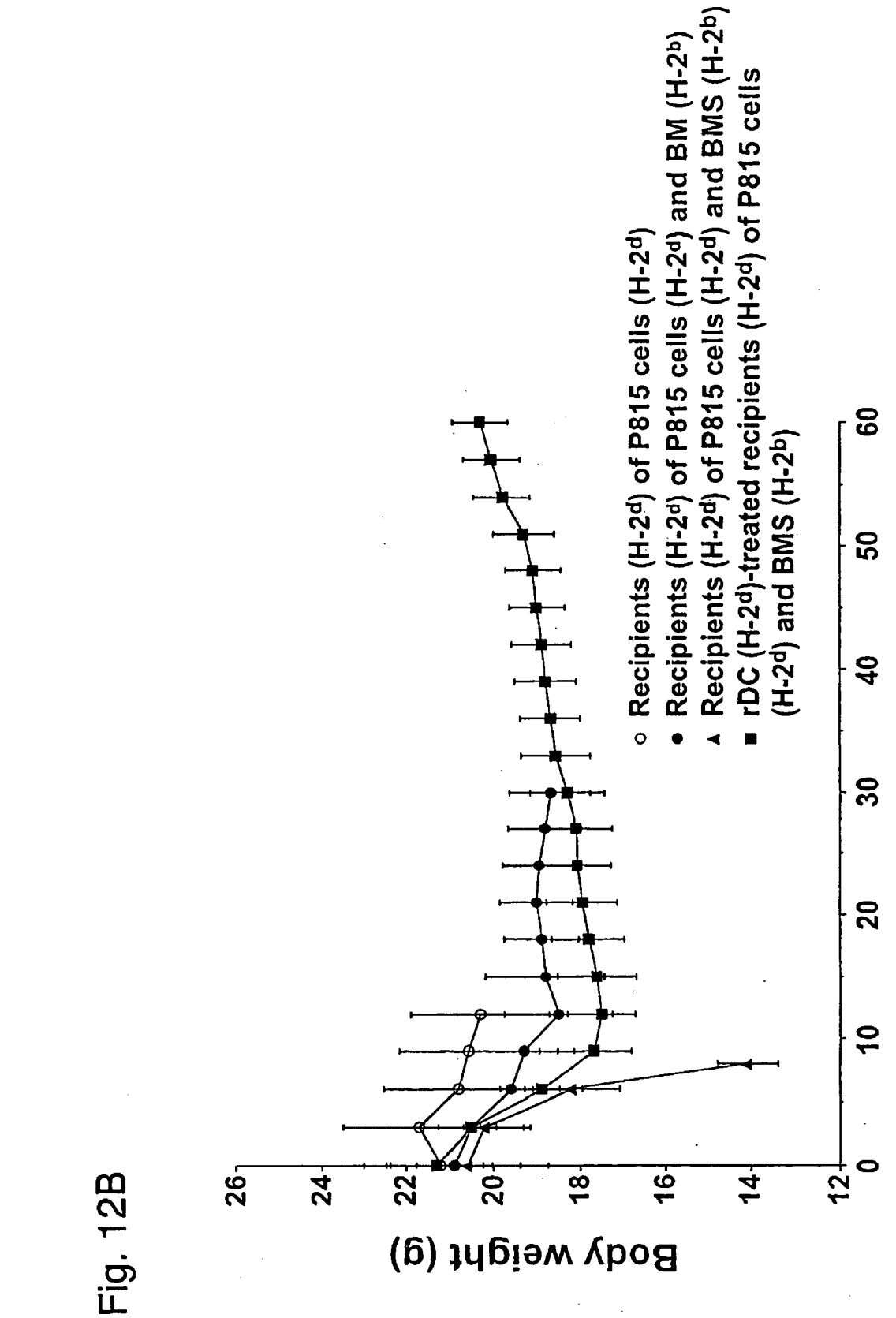

Body weight (g)
26
24
22
20
18
16
14
12
0
10
20
30
40
50
60
Days after transplantation
○ Recipients (H-2d) of P815 cells (H-2d)
● Recipients (H-2d) of P815 cells (H-2d) and BM (H-2b)
▲ Recipients (H-2d) of P815 cells (H-2d) and BMS (H-2b)
■ rDC (H-2d)-treated recipients (H-2d) of P815 cells (H-2d) and BMS (H-2b)

Fig. 12B

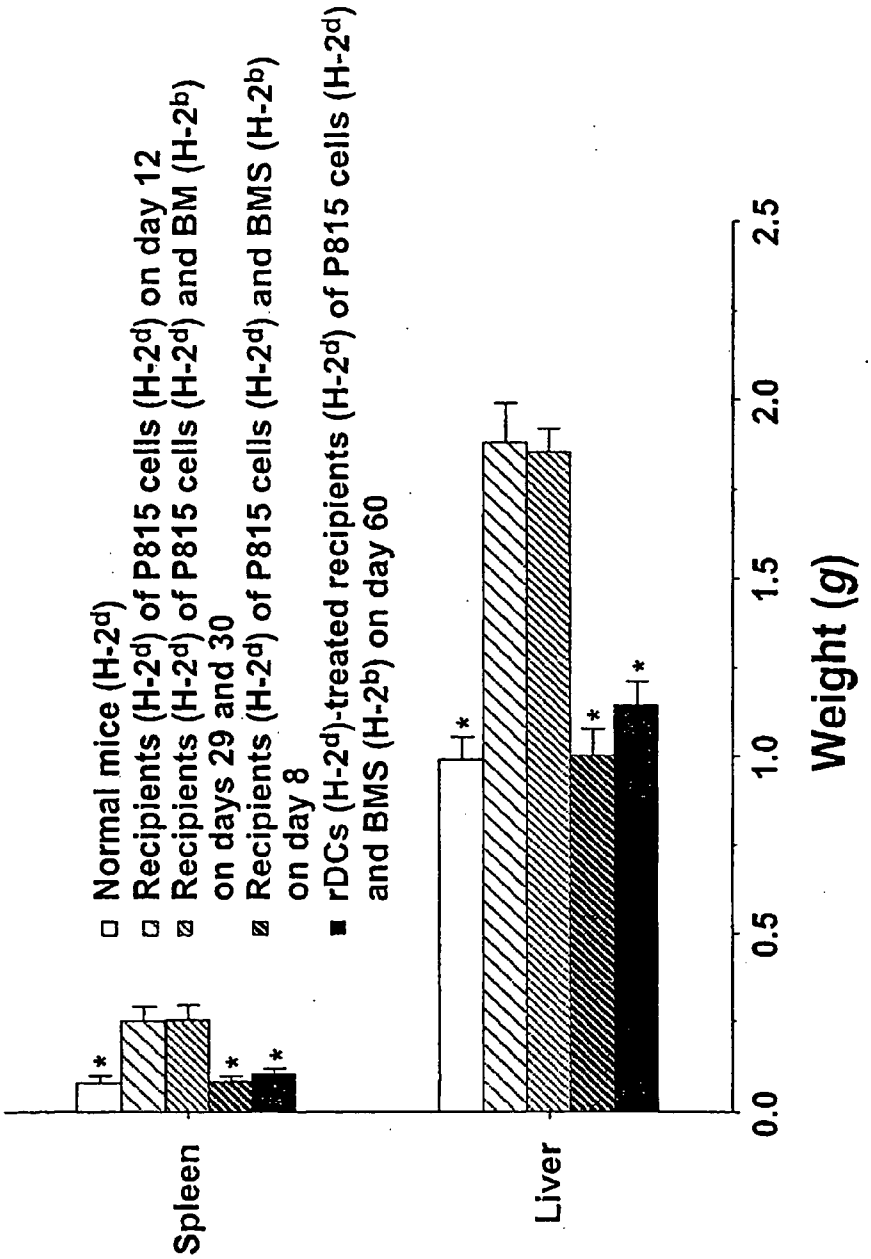

Normal mice (H-2d)
Recipients (H-2d) of P815 cells (H-2d) on day 12
Recipients (H-2d) of P815 cells (H-2d) and BM (H-2b) on days 29 and 30
Recipients (H-2d) of P815 cells (H-2d) and BMS (H-2b) on day 8
rDCs (H-2d)-treated recipients (H-2d) of P815 cells (H-2d) and BMS (H-2b) on day 60
Spleen
Liver
0.0
0.5
1.0
1.5
2.0
2.5
Weight (g)

Fig. 12C

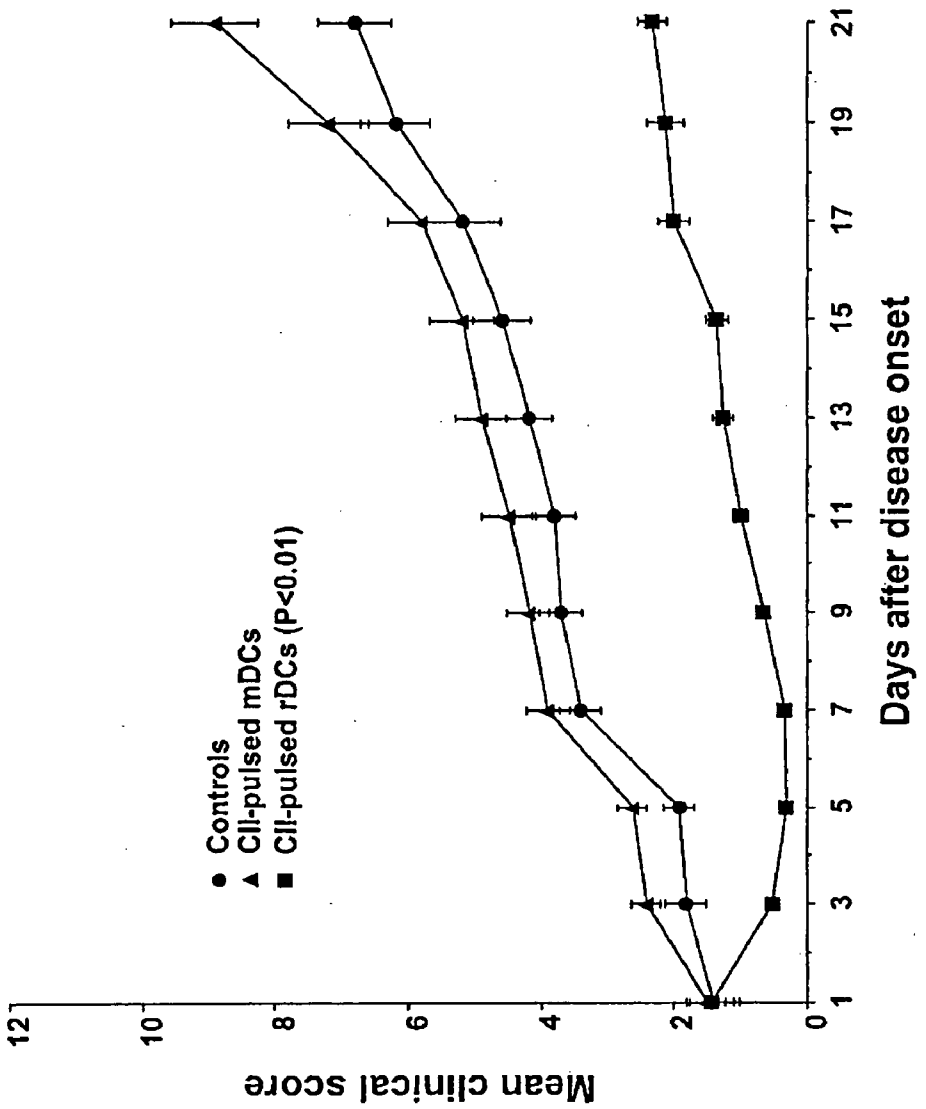

Mean clinical score
Days after disease onset
Controls
CII-pulsed mDCs
CII-pulsed rDCs (P<0.01)
0
2
4
6
8
10
12
1
3
5
7
9
11
13
15
17
19
21

Fig. 13A

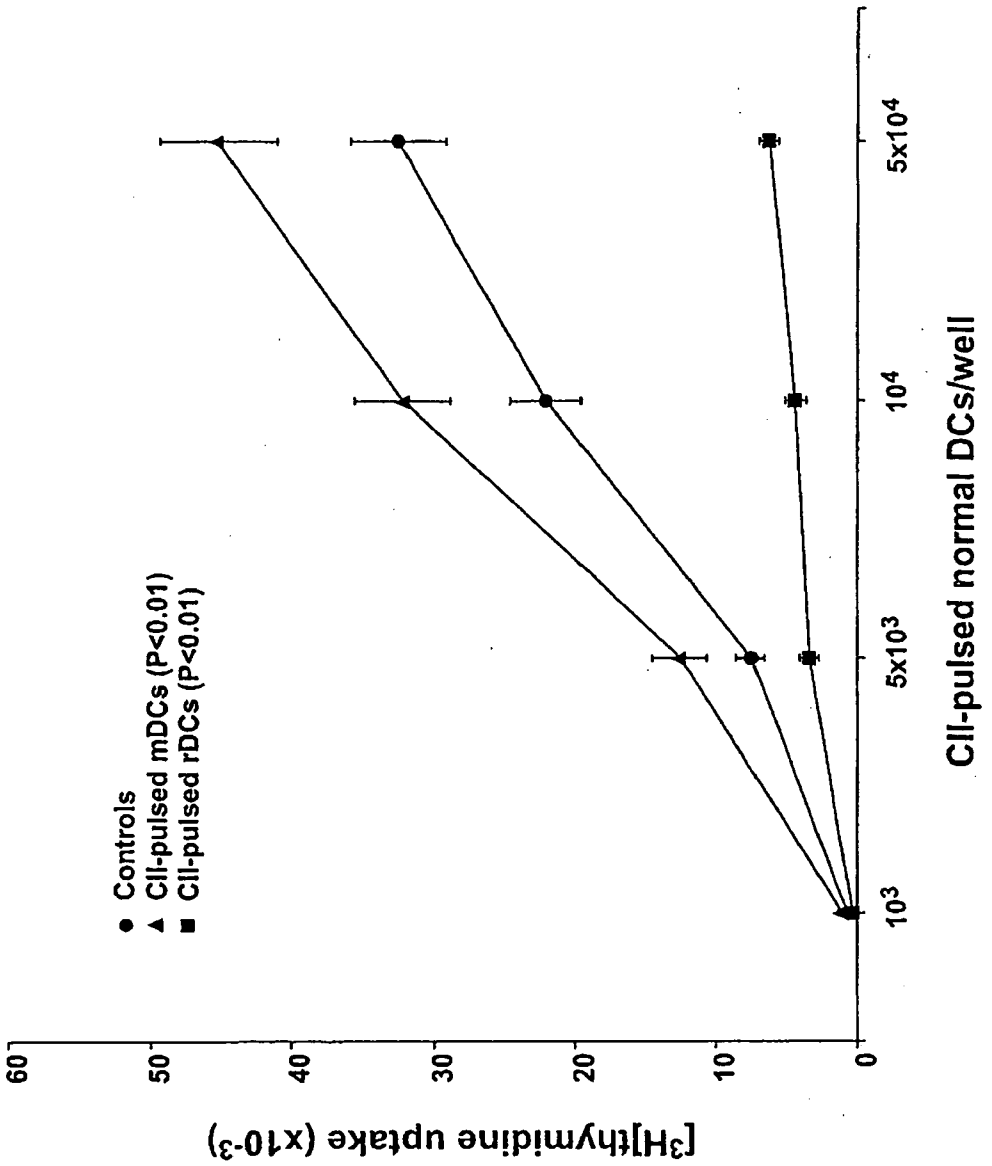

[3H]thymidine uptake (x10-3)
60
50
40
30
20
10
0
10^3
5x10^3
10^4
5x10^4
CII-pulsed normal DCs/well
Controls
CII-pulsed mDCs (P<0.01)
CII-pulsed rDCs (P<0.01)

Fig. 13B

Fig.14

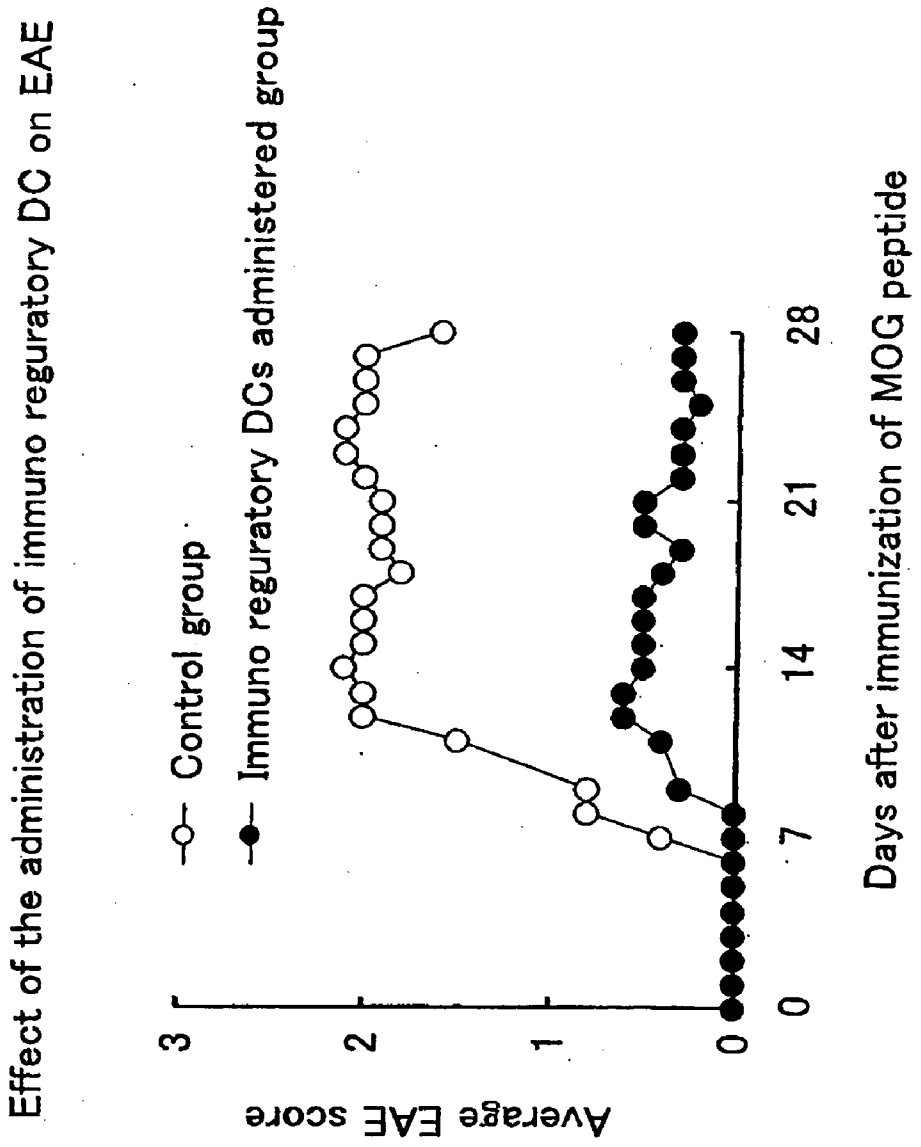
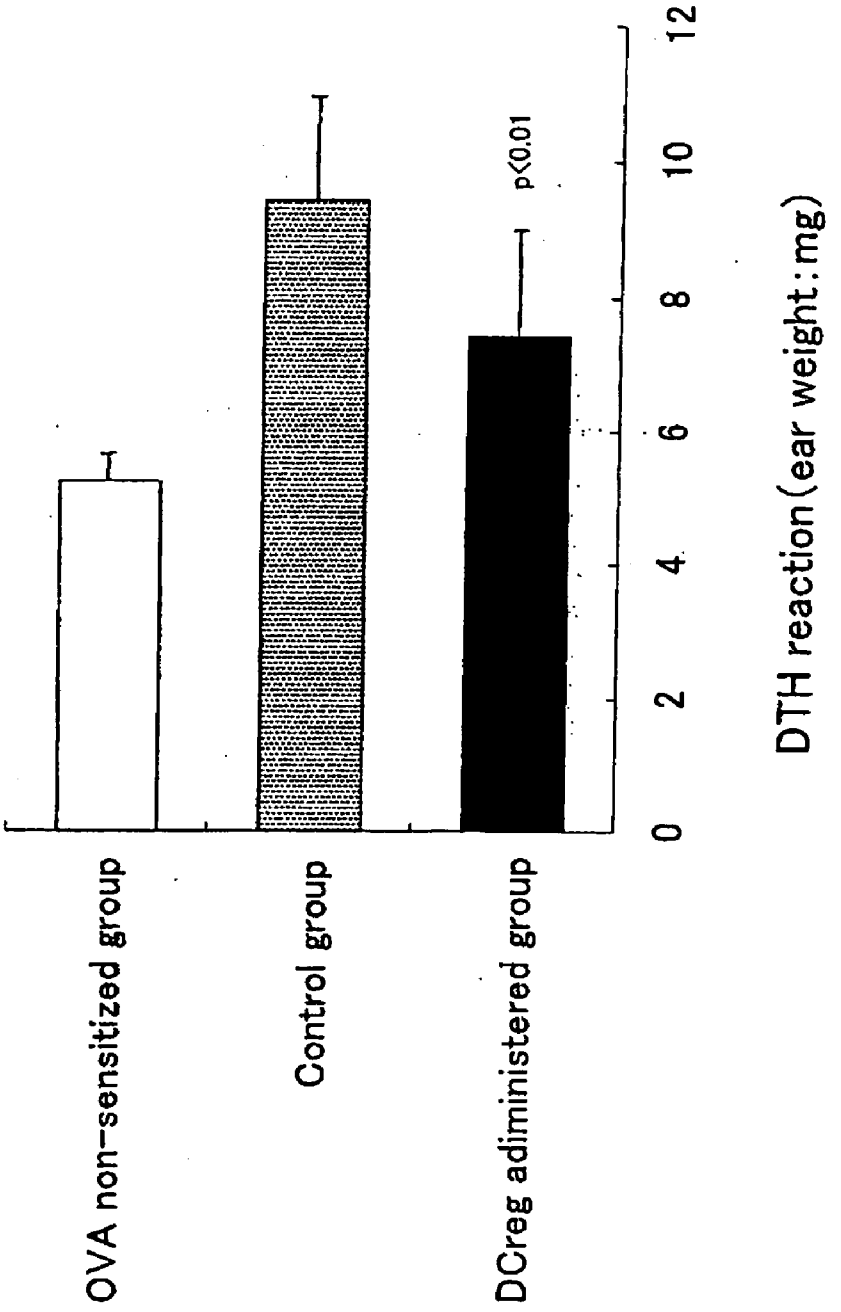

Effect of the administration of immuno reguratory DC on EAE
Control group
Immuno reguratory DCs administered group
Average EAE score
3
2
1
0
0
7
14
21
28
Days after immunization of MOG peptide

Fig.15


DTH reaction (ear weight: mg)
0
2
4
6
8
10
12
p<0.01
OVA non-sensitized group
Control group
DCreg adiministered group

METHOD OF PREPARING IMMUNO-REGULATORY DENDRITIC CELLS AND THE USE THEREOF

TECHNICAL FIELD

[0001] The present invention relates to a therapeutic agent for graft rejection, graft-versus-host disease, autoimmune disease, allergic disease, or other diseases comprising dendritic cells (DCs) induced under culture conditions comprising at least both of IL-10 and TGF-β or DCs prepared by adding inflammatory stimulation (e.g., TNF-α or LPS) to the aforementioned DCs and, if necessary, an antigen associated with a target disease.

BACKGROUND ART

[0002] Dendritic cells (DCs) are the most potent antigen-presenting cells in an organism, and they are known to induce immune responses by presenting an antigen to T cells. DCs are known to act directly not only on T cells but also on B cells, NK cells, NKT cells, and other cells, and they play major roles in immune reactions (Hart, D. N. J., Blood 1997, 90: 3245-3278). Immature DCs exist in peripheral tissues, and they arc highly capable of incorporating antigens, although their capability of stimulating T cells is low. When immature DCs receive infectious or inflammatory stimuli, they cause costimulating molecules such as CD40, CD80, or CD86 to be expressed with higher frequency, and they acquire high capability of stimulating T cells. At the same time, they are transferred to peripheral lymphatic tissues, and they activate T cells specific to the antigens incorporated, thereby inducing immune responses (Banchereau, J. et al., Annu. Rev. Immunol. 2000, 18: 767-811). A technique of inducing DCs in vitro is being established, and cancer-specific antigens have been successively identified. Based on these achievements, research aimed at the application of the potent capability of DCs in terms of inducing immunity to the treatment of cancer is expanding. Such novel cellular medicine has drawn attention as future medicine, and development thereof is expected.

[0003] In a healthy organism, mechanisms of immune response function against foreign ("nonself") antigens or tumors to eliminate them. However, immune tolerance to normal self-antigens constituting an organism or harmless foreign antigens can be established, and eliminative mechanisms of immune responses may not function against them. Regulatory mechanisms against autoimmune, allergic, or other diseases, which are disturbed for any reason, are considered to cause such diseases. DCs play major roles not only as antigen presenting cells in the establishment of immune responses against infections or cancer, but they also play important roles in induction of immune tolerance (Steinman, R. M., Proc. Natl. Acad. Sci. USA 2002, 99: 351-358). Immune tolerance is roughly classified into two types: elimination of self-reactive T cell clones in thymic glands, which is referred to as central tolerance; and regulation of self-reactive T cells outside the thymic glands, which is a mechanism referred to as peripheral tolerance. In particular, the latter is known to induce cell death or anergy to self-antigens and to have active suppressing mechanisms mediated by immunoregulatory T cells (Roncarolo, M. G. and Levings M. K., 2000, Curr. Opinion Immunol. 12: 676-683) DCs have been found to be capable of inducing cell death and anergy to T cells and to be capable of inducing immunoregulatory T cells. Clarification of the way that DCs acquire such functional multidimensionality for immune response regulation is making progress in terms of, for example, differences in the maturation phase of DCs, existence of a subset of DCs with different functions, and types of stimuli such as cytokines or pathogens. Based on these findings, application of the capability of DCs to induce immune tolerance to the treatment of autoimmune disease or suppression of graft rejection has been examined (Jonulcit, H. et al., Trends in Immunol. 2001, 22: 394-400; Hackstein, H. et al., Trends in Immunol. 2001, 22: 437-442).

[0004] Immature DCs induced from human monocytes with the aid of GM-CSF and IL-4 were further cultured in GM-CSF and IL-b10 for 2 days. In the thus prepared DCs, expression levels of costimulating molecules CD58, CD86, and CD83 are lower, and growth of $CD4^+$ T cells caused by an allogeneic mixed leukocyte reaction (allo-MLR) is suppressed (Steinbrink, K. et. al., J. Immunol. 1997, 159: 4772-4780). Immature DCs induced from human monocytes with the aid of GM-CSF and IL-4 were further cultured in GM-CSF and IL-10 for 2 days, and the thus prepared DCs induce $CD4^+$ and $CD8^+$ T cells that are anergic against antigenic stimuli and have immune-suppressing activity (Steinbrink, K. et. al., Blood 2002, 99: 2468-2476). When naive $CD4^+$ T cells are stimulated repeatedly with immature human DCs, $CD4^+$ $CD25^+$ immunoregulatory T cells highly capable of producing IL-10 are induced. These T cells suppress antigen-specific growth of activated Th1 cells (type 1 helper T cells) and production of cytokines in vitro (Jonuleit, H. et al., J. Exp. Med. 2000, 192: 1213-1222). Administration of immature human DCs comprising a peptide derived from the influenza virus matrix protein to a healthy individual resulted in suppression of the aforementioned antigen-specific $CD8^+$ T cells and in induction of the aforementioned antigen-specific $CD8^+$ IL-10-producing immunoregulatory T cells. This suppressing effect, however, disappeared 6 months after administration of DCs (Dhodapkar, M. V. et al., J. Exp. Med. 2001, 193: 233-238; Dhodapkar, M. V. et al., Blood 2002, 100: 174-177). Thus, it is suggested that immature DCs induced from human monocytes with the aid of GM-CSF and IL-4 or DCs maintain their immature states by being treated with IL-10 induce antigen-specific immune suppression. Under inflammatory conditions such as those of autoimmune diseases, however, immature DCs are induced to mature, and whether or not DCs are capable of maintaining immunoregulatory functions is an issue in question.

[0005] Murine bone marrow-derived DCs induced with the aid of GM-CSF and TGF-β1 have features of immature DCs and have attenuated activity for accelerating growth of allogeneic and naive $CD4^+$ T cells (Yamaguchi, Y. et al., Stem Cells. 1997, 15(2): 144-53). The aforementioned DCs were administered to a mouse allogeneic heart transplant model to prolong graft survival (Lu, L. et. al., Transplantation 1997, 64: 1808-1815). Similarly, GM-CSF-induced murine bone marrow-derived immature DCs and GM-CSF-induced murine liver-derived immature DCs exhibited effects of prolonging graft survival in an allogeneic transplant model (Lutz, M. et al., Eur. J. Immunol. 2000, 30: 1813-1822; Fu, F. et al., Transplantation 1996, 62: 659-665; Rastellini, C. et al. Transplantation 1995, 60: 1366-1370). In contrast, murine spleen-derived $CD8^+$ DCs exhibited effects of prolonging graft survival in an allogeneic transplant

model regardless of its maturation phase (O'Connell, P. J. et al. J. Immunol. 2002, 168: 143-154). The effects of suppressing autoimmune diabetes in a NOD mouse were observed only when mature DCs were administered instead of immature DCs (Feili-Hariri, M. et al., Eur. J. Immunol. 2002, 32: 2021-2030). In the case of EAE multiple sclerosis models, effects of suppressing the target disease by semi-mature DCs treated with TNF-α for a short period of time have been reported (Menges, M. et al., J. Exp. Med. 2002, 195: 15-21). Thus, it is difficult to judge the immune-suppressing properties of DCs depending on their maturation phases in research utilizing murine DCs. Concerning murine DCs, effects of suppressing a target disease in a transplant model to which a gene of a molecule associated with suppression and regulation of immune responses, such as FasL, CTLA-4-Ig, IL-10, TGF-β, and IL-4, have been introduced and in an autoimmune disease model have been reported (Hackstein, H. et al., Trends in Immunol. 2001, 22: 437-442). DCs induced from murine bone marrow cells with the aid of GM-CSF and IL-4 were used, IL-10-transduced DCs and TGF-β-transduced DCs were mixed with each other, and the resultant was administered in the portal vein. The effects thereof for prolonging graft survival were observed in an allogeneic kidney transplant model (Gorczynski, R. M. et al., Clin. Immunol. 2000, 95: 182-189).

[0006] List of References

[0007] Jonuleit, H. et al., Trends in Immunol. 2001, 22: 394-400

[0008] Hackstein, H. et al., Trends in Immunol. 2001, 22: 437-442

[0009] Steinbrink, K. et al., J. Immunol. 1997, 159: 4772-4780

[0010] Steinbrink, K. et al., Blood 2002, 99: 2468-2476

[0011] Jonuleit, H. et al., J. Exp. Med. 2000, 192: 1213-1222

[0012] Dhodapkar, M. V. et al., J. Exp. Med. 2001, 193: 233-238

[0013] Dhodapkar, M. V. et al., Blood 2002, 100: 174-177

[0014] Yamaguchi, Y. et al., Stem Cells. 1997, 15 (2): 144-53

[0015] Lutz, M. et al., Eur. J. Immunol. 2000, 30: 1813-1822

[0016] Fu, F. et al., Transplantation 1996, 62: 659-665

[0017] Rastellini, C. et al. Transplantation 1995, 60: 1366-1370

[0018] O'Connell, P. J. et al. J. Immunol. 2002, 168:143-154

[0019] Feili-Hariri, M. et al., Eur. J. Immunol. 2002, 32: 2021-2030

[0020] Menges, M. et al., J. Exp. Med. 2002, 195: 15-21

[0021] Hackstein, H. et al., Trends in Immunol. 2001, 22: 437-442

[0022] Gorczynski, R. M. et al., Clin. Immunol. 2000, 95:182-189

DISCLOSURE OF THE INVENTION

[0023] Objects of the present invention are to provide human immunoregulatory dendritic cells having immunoregulatory properties even under inflammatory disease conditions, a method of inducing the immunoregulatory dendritic cells, and a pharmaceutical composition comprising the immunoregulatory dendritic cells. More particularly, objects of the present invention are to provide a method of culturing human dendritic cells or their precursor cells in the presence of cytokines comprising at least IL-10 and TGF-β, thereby inducing human immunoregulatory dendritic cells, human immunoregulatory dendritic cells induced by the aforementioned method, and the use of the aforementioned immunoregulatory dendritic cells for treating graft rejection, graft-versus-host disease, autoimmune disease, and allergic disease.

[0024] As mentioned above, attempts have been heretofore made to obtain dendritic cells (DCs) having immune-suppressing activity. These dendritic cells, however, merely maintained their immature states, and maturation thereof was disadvantageously induced under inflammatory conditions. Thus, whether or not they could maintain immune-suppressing activity was an issue of concern. The present inventor has conducted concentrated studies in order to induce dendritic cells that can sufficiently function even under inflammatory disease conditions. As a result, he has found that immunoregulatory DCs induced with the aid of IL-10 in combination with TGF-β have effects of inducing immune responses and potently suppressing the target disease in a disease model. Further, the present inventor has examined the usefulness of the aforementioned immunoregulatory DCs in terms of treatment of graft rejection or diseases associated with immunity, and as a result, he has found that the DCs have effects of suppressing graft rejection and treating immune-associated diseases. This has led to the completion of the present invention.

[0025] More specifically, the present invention is as follows:

[0026] [1] a method of preparing human immunoregulatory dendritic cells by culturing human dendritic cells or their precursor cells in vitro with IL-10 and TGF-β;

[0027] [2] the method of preparing human immunoregulatory dendritic cells according to [1], wherein the human dendritic cells are derived from human monocytes;

[0028] [3] a method of preparing human immunoregulatory dendritic cells comprising culturing human monocytes in the presence of GM-CSF, IL-4, IL-10, and TGF-β;

[0029] 4] the method of preparing human immunoregulatory dendritic cells according to [3], wherein culture is further conducted in the presence of at least one of TNF-β and LPS;

[0030] [5] the method of preparing human immunoregulatory dendritic cells according to any of [1] to [4], wherein culture is further conducted in the presence of an antigen existing in a tissue or organ associated with a disease to be treated;

[0031] [6] the method of preparing human immunoregulatory dendritic cells according to [5], wherein the disease is an autoimmune or allergic disease;

[0032] [7] The method of preparing human immunoregulatory dendritic cells according to [5], wherein the disease is rheumatoid arthritis or multiple sclerosis.

[0033] [8] human immunoregulatory dendritic cells prepared by the method according to any of [1] to [7];

[0034] [9] the human immunoregulatory dendritic cells according to [8], wherein expression levels of CD83, CD40, CD80, and CD86 are significantly lower than those in mature human dendritic cells that were not cultured in the presence of both IL-10 and TGF-β;

[0035] [10] the human immunoregulatory dendritic cells according to [8] or [9], which are capable of inducing antigen-specific anergy to allogeneic $CD4^+$ T cells in vitro, suppressing reactivation of activated allogeneic $CD4^+$ T cells and $CD8^+$ T cells, inducing allogeneic and naive $CD4^+$ T cells and $CD8^+$ T cells to become $CD4^+$ $CD25^+$ immunoregulatory T cells and $CD8^+$ $CD28^-$ immunoregulatory T cells, respectively, and inducing immune-suppressing responses such as the suppression of graft-versus-host disease after xenogeneic transplantation in a human T cell-transplanted imnmunodeficient mouse through administration of the aforementioned cells to which xenoantigens have been imparted;

[0036] [11] a pharmaceutical composition comprising the human immunoregulatory dendritic cells according to any of [8] to [10];

[0037] [12] the pharmaceutical composition according to [11], which suppresses graft rejection caused along with cell, organ, or tissue transplantation;

[0038] [13] the pharmaceutical composition according to [11], which can be used for treating graft-versus-host disease; [14] the pharmaceutical composition according to [11], which can be used for treating an autoimmune or allergic disease; and

[0039] [15] The pharmaceutical composition according to [11], which can be used for treating rheumatoid arthritis or multiple sclerosis.

[0040] The present invention is hereafter described in detail.

[0041] 1. Preparation of Human Immunoregulatory DCs

[0042] The present invention relates to a method of preparing human immunoregulatory DCs by culturing human dendritic cells (DCs) or their precursor cells in the presence of cytokines comprising at least IL-10 and TGF-β and to human immunoregulatory DCs obtained by the aforementioned method. For example, GM-CSF, IL-4, IL-10, or TGF-β1 is added to human monocytes to induce DCs, and inflammatory stimuli (such as TNF-α or LPS) arc further added to the thus prepared DCs to induce another type of DCs. The thus obtained DCs have human immunoregulatory properties. Human monocytes arc cultured in vitro in the presence of GM-CSF and IL-4, human monocytes are then differentiated to result in DCs, and DCs become immature immunoregulatory DCs with the aid of IL-10 and TGF-β. In such a case, human monocytes may be first stimulated with GM-CSF and IL-4 for differentiation to DCs, followed by stimulation with IL-10 and TGF-β. Alternatively, human monocytes may be simultaneously stimulated with GM-CSF, IL-4, IL-10, and TGF-β1. Further, immature human immunoregulatory DCs become mature human immunoregulatory cells through application of inflammatory stimuli such as TNF-α or LPS. Furthermore, when human dendritic cells (DCs) or their precursor cells are cultured in the presence of cytokines comprising at least IL-10 and TGF-β to prepare human immunoregulatory DCs, CD40 agonist may be added to the culture. CD40 agonist means a substance which acts on CD40 antigen expressed on the surface of an immune cell and transmits a signal into the cell via CD40. CD40 agonist includes natural or synthetic ligand, i.e. any molecule which transmits a signal via CD40, and an antibody against CD40 antigen. The antibody may recognize any site of CD40 as long as it induces a signal via CD40. It has been reported that anti CD40 antibody matures DCs (Z. H. Zhou et al., Hybridoma, 18:471 1999). The antibody used for the present inventions is not limited to but preferably is the antibody disclosed in WO 2002/099196. An antibody fragment which maintains an antigen-recognizing site of the antibody is also useful as CD40 agonist.

[0043] Human DCs can be obtained by culturing human monocytes in the presence of GM-CSF and IL-4 as described above. In this case, monocytes may be derived from human peripheral blood, human bone marrow, human spleen cells, or human umbilical cord blood. Further, dendritic cells can be isolated from these tissues or organs using a fluorescent activated cell sorter (FACS) or a flowcytometer while employing expression of DC-specific surface antigen, such as CD1a, as an indicator. A specific cell group can be isolated using FACS in accordance with a known technique. Examples of FACS and a flowcytometer that can be used are the FACSVantage (Becton Dickinson) and FACSCalibur (Becton Dickinson).

[0044] Human monocytes and DCs can be cultured in accordance with a known culture technique for human lymphoid cells. For example, RPMI 1640 or DMEM can be used as a culture medium, and adequate antibiotics or animal serum may be added to such basal medium to conduct culture. Also, a culture vessel is not particularly limited, and a commercialized plate, dish, or flask can be adequately selected in accordance with a scale of culture.

[0045] Concentration of GM-CSF, IL,-4, IL-10, TGF-β1, TNF-α, or LPS for culture is 1 ng/ml to 1,000 ng/ml, and preferably 10 ng/ml to 100 ng/ml. In case where CD40 agonist, for example anti-CD40 antibody, is added, concentration of anti CD40 antibody is 0.1 μg/mL to 100 μg/mL, and preferably 1 μg/mL to 10 μg/mL. The number of days necessary for stimulation is not limited. For example, human monocytes may be cultured together with GM-CSF, IL-4, IL-10, TGF-β1, TNF-α, or LPS for periods of several days to about 10 days. Inspection of expression of human monocytes or human DC surface antigen by FACS or other means enables determination of a suitable culture period for obtaining cells with a differentiation level of interest. Conditions such as concentration of GM-CSF, IL-4, IL-10, TGF-β1, TNF-α, or LPS for stimulation or a period of stimulation can

be determined while employing induction of antigen-specific anergy to allogeneic $CD4^+$ T cells or DC phenotypes as indicators.

[0046] Human immunoregulatory DCs are capable of inducing antigen-specific anergy to allogeneic $CD4^+$ T cells in vitro, suppressing reactivation of activated allogeneic $CD4^+$ T cells and $CD8^+$ T cells, inducing allogeneic and naive $CD4^+$ T cells and $CD8^+$ T cells to become $CD4^+$ $CD25^+$ immunoregulatory T cells and $CD4^+$ $CD28^-$ immunoregulatory T cells, respectively, and inducing immune-suppressing responses such as the suppression of graft-versus-host disease after xenogeneic transplantation in a human T cell-transplanted immunodeficient mouse through administration of the aforementioned cells to which xenoantigens have been imparted. Whether or not cells have such features can be determined by the method described in the Examples below.

[0047] These human immunoregulatory DCs exhibit similar functions even when they were treated with inflammatory cytokines such as TNF-α in addition to immature cells. This indicates that these DCs can function sufficiently even under inflammatory disease conditions. Specifically, the immunoregulatory DCs of the present invention include both mature and immature DCs. Whether a DC is mature or immature can be determined by, for example, inspecting the expression of CD83 on the surface thereof. CD83 is expressed on the surface of a mature DC.

[0048] 2. Use of Human Immunoregulatory DCs

[0049] As mentioned above, the human immunoregulatory DCs of the present invention are capable of inducing antigen-specific anergy to allogeneic CD4+ T cells in vitro, suppressing reactivation of activated allogeneic $CD4^+$ T cells and $CD8^+$ T cells, inducing allogeneic and naive $CD4^+$ T cells and $CD8^+$ T cells to become $CD4^+$ $C25^+$ immunoregulatory T cells and $CD8^+$ $CD28^-$ immunoregulatory T cells, respectively, and inducing immune-suppressing responses such as the suppression of graft-versus-host disease after xenogeneic transplantation in a human T cell-transplanted immunodeficient mouse through administration of the aforementioned cells to which xenoantigens have been imparted.

[0050] As with the case of human immunoregulatory dendritic cells, murine immunoregulatory DCs induced with the aid of IL-10 in combination with TGF-β1 exhibited effects of suppressing graft-versus-host disease after allogeneic transplantation while maintaining the graft-versus-leukemia effects and effects of suppressing the developed disease in a murine autoimmune arthritis model, in addition to the effects of suppressing graft-versus-host disease after allogeneic transplantation and graft-versus-host disease after xenogeneic transplantation. Functions of these murine immunoregulatory DCs are equivalent to those of the aforementioned human immunoregulatory DCs in the following respects, and human dendritic cells were suggested to be effective for treatment of diseases presented in the case of murine immunoregulatory DCs: 1) the phenotype of a cell surface molecule: a costimulating molecule (CD40, CD80, or CD86) being expressed at low frequency and an MHC molecule being expressed; 2) induction of antigen-specific anergy to allogeneic $CD4^+$ T cells; 3) suppression of reactivation of activated allogeneic $CD4^+$ T cells; 4) suppression of graft-versus-host disease after xenogeneic transplantation in a human T cell-transplanted immunodeficient mouse; 5) induction of $CD4^+$ $CD25^+$ $CD152^+$ T cells in vitro; and 6) the fact that human immunoregulatory DCs induce cells similar to $CD4^+$ $CD25^+$ immunoregulatory T cells involved in the suppression of graft-versus-host disease after allogeneic transplantation caused by murine immunoregulatory DCs in vitro.

[0051] As a conventional technique, immunoregulatory DCs induced with the aid of IL-10 or TGF-β alone had been discovered. The human immunoregulatory DCs of the present invention induced with the aid of IL-10 in combination with TGF-β more significantly induced antigen-specific anergy to allogeneic $CD4^+$ T cells compared with DCs induced with the aid of a cytokine alone. This indicates that the human immunoregulatory DCs of the present invention have more significant therapeutic effects compared with those attained by DCs induced with the aid of a cytokine alone.

[0052] As is apparent from the foregoing description, human immunoregulatory DCs can suppressively regulate immune responses of $CD4^+$ or $CD8^+$ T cells. Accordingly, the human immunoregulatory DCs of the present invention can be used for novel treatment of a variety of diseases caused by immune reactions associated with $CD4^+$ or $CD8^+$ T cells.

[0053] Disease to be treated in the present invention includes an organ specific autoimmune disease associated with delayed type hypersensitivity (DTH). The delayed type hypersensitivity (DTH) is a typical Th1 response and considered to be a basic reaction for chronic inflammation in the organ specific autoimmune disease. The organ specific autoimmune diseases associated with Th1 immune response mainly include multiple sclerosis, rheumatism, type I diabetes, uveitis, autoimmune myocarditis and Crohn's disease, and include contact hypersensitivity as allergic diseases.

[0054] Furthermore, diseases to be treated in the present invention are: in addition to graft rejection caused along with cell, organ, or tissue transplantation and graft-versus-host disease, autoimmune diseases such as rheumatoid arthritis, multiple sclerosis, type I diabetes, uveitis, autoimmune myocarditis, myasthenia gravis, systemic erythematodes, autoimmune hemolytic anemia, systemic scleroderma, ulcerous colitis, Crohn's disease, Sjogren's syndrome, autoimmune hepatopathy (e.g., primary biliary cirrhosis), psoriasis, idiopathic thrombocytopenic purpura, Goodpasture syndrome (e.g., glomerular nephritis), pernicious anemia, Hashimoto's disease, vitiligo vulgaris, Behcet's disease, autoimmune gastritis, pemphigus, Guillain-Barre syndrome, and HTLV-1-associated myelopathy; and allergic diseases such as contact hypersensitivity, allergic rhinitis, food allergies, and asthma.

[0055] The present invention includes a pharmaceutical composition for treating the aforementioned diseases comprising the human immunoregulatory DCs induced by the method of the present invention. When the human immunoregulatory DCs of the present invention are used for treating diseases, they are stimulated with an antigen associated with the disease to be treated. In the case of autoimmune disease or allergic diseases, antigen proteins or peptides existing in tissues or organs associated with the disease, RNA or DNA encoding them, or modified forms thereof are used as antigens. In the case of graft rejection or

graft-versus-host disease, application of an antigen is not necessary since DCs have internally expressed allogeneic antigen. Alternatively, a donor- or recipient-derived antigen may be used. As stimulation in such a case, the human immunoregulatory DCs of the present invention may be cultured in vitro together with an antigen.

[0056] Human DCs for a pharmaceutical composition for treatment are human monocyte-derived DCs (Bwatricc Thurner, Gerold Schuler et al, J. Exp. Med. 1999, 190 (11): 1669-1678; Axel Heiser, Eli Gilboa el al, J. Clin. Invest. 2002, 109 (3): 409-417), human peripheral blood-derived DCs (Small E J., L Clin Oncol. 2000, 18 (23): 3894-3903), or human $CD34^+$ cell-derived DCs (Caux C, Jacques Banchereau et al Blood 1997, 90 (4): 1458-1470), with human monocyte-derived DCs being preferable.

[0057] When treating autoimmune or allergic diseases, antigens are imparted to DCs in vitro for 1 to 10 days including the final day of culture at concentrations of 1 to 10mg/ml, and preferably 10 ng/ml to 5 mg/ml in the case of protein antigens. When human immunoregulatory DCs that were further stimulated with inflammatory stimuli such as TNF-α or LPS are used, antigens are preferably imparted simultaneously with or prior to the application of stimuli.

[0058] When a pharmaceutical composition comprising the human immunoregulatory DCs of the present invention is used for treatment, this composition is intravenously, subcutaneously, or intracutancously (preferably intravenously) administered in amounts of 0.5×10^5 to 10^9 in terms of each DC fraction.

[0059] The pharmaceutical composition can be administered to a patient on an as-needed basis (preferably during the symptom-free period). In the case of graft rejection and graft-versus-host disease caused along with organ or tissue transplantation, the compound is preferably administered to a patient before the treatment that is supposed to result in the development of disease.

[0060] The timing of administration and the dose of human immunoregulatory DCs can be adequately determined in accordance with, for example, the type of disease, the severity of disease, and the conditions of a patient.

[0061] The present invention includes a method of treating an organ specific autoimmune disease associated with delayed type hypersensitivity (DTH) comprising administration of the human immunoregulatory DCs of the present invention. The delayed type hypersensitivity (DTH) is a typical Th1 response and considered to be a basic reaction for chronic inflammation in the organ specific autoimmune disease. The organ specific autoimmune diseases associated with Th1 immune response mainly include multiple sclerosis, rheumatism, type I diabetes, uveitis, autoimmune myocarditis and Crohn's disease, and include contact hypersensitivity as allergic diseases. Further, the present invention includes a method of treating diseases comprising administration of the human immunoregulatory DCs of the present invention. Diseases to be treated by the present invention are: in addition to graft rejection and graft-versus-host disease caused along with cell, organ, or tissue transplantation, autoimmune diseases such as rheumatoid arthritis, multiple sclerosis, type I diabetes, uveitis, autoimmune myocarditis, myasthenia gravies, systemic crythematodes, autoimmune hemolytic anemia, systemic scleroderma, ulcerous colitis, Crohn's disease, Sjogren's syndrome, autoimmune hepatopathy (e.g., primary biliary cirrhosis), psoriasis, idiopathic thrombocytopenic purpura, Goodpasture syndrome (e.g., glomerular nephritis), pernicious anemia, Hashimoto's disease, vitiligo vulgaris, Behcet's disease, autoimmune gastritis, pemphigus, Guillain-Barre syndrome, and HTLV-1-associated myelopathy; and allergic diseases such as contact hypersensitivity, allergic rhinitis, food allergies, and asthma. In this case, human immunoregulatory DCs to be administered to a patient may be prepared by stimulating monocytes or DCs of the patient in vitro or stimulating monocytes or DCs of an unrelated individual other than the patient in vitro. The present invention also includes the use of the human immunoregulatory DCs of the present invention for preparing therapeutic agents for the aforementioned diseases.

[0062] This description includes part or all of the contents as disclosed in the description and/or drawings of Japanese Patent Application No. 2003-073799, which is a priority document of the present application.

BRIEF DESCRIPTION OF THE DRAWINGS

[0063] **FIG. 1A** shows phenotypes of modified human DCs.

[0064] **FIG. 1B** shows phenotypes of modified human DCs.

[0065] **FIG. 1C** shows phenotypes of modified human DCs.

[0066] **FIG. 1D** shows phenotypes of modified human DCs.

[0067] **FIG. 2A** shows that, among modified human DCs, IL-10/TGF-β1-induced DCs function as immunoregulatory DCs to induce antigen-specific anergy to human T cells and suppress reactivation of activated T cells.

[0068] **FIG. 2B** shows that, among modified human DCs, IL-10/TGF-β1-induced DCs function as immunoregulatory DCs to induce antigen-specific anergy to human T cells and suppress reactivation of activated T cells.

[0069] **FIG. 2C** shows that, among modified human DCs, IL-10/TGF-β1-induced DCs function as immunoregulatory DCs to induce antigen-specific anergy to human T cells and suppress reactivation of activated T cells.

[0070] **FIG. 2D** shows that, among modified human DCs, IL-10/TGF-β1-induced DCs function as immunoregulatory DCs to induce antigen-specific anergy to human T cells and suppress reactivation of activated T cells.

[0071] **FIG. 2E** shows that, among modified human DCs, IL-10/TGF-β1-induced DCs function as immunoregulatory DCs to induce antigen-specific anergy to human T cells and suppress reactivation of activated T cells.

[0072] **FIG. 2F** shows that, among modified human DCs, IL-10/TGF-β1-induced DCs function as immunoregulatory DCs to induce antigen-specific anergy to human T cells and suppress reactivation of activated T cells.

[0073] **FIG. 2G** shows that, among modified human DCs, IL-10/TGF-β1-induced DCs function as immunoregulatory DCs to induce antigen-specific anergy to human T cells and suppress reactivation of activated T cells.

[0074] **FIG. 3A** shows that human immunoregulatory DCs induce $CD4^+$ $CD25^+$ human immunoregulatory T cells.

[0075] **FIG. 3B** shows that human immunoregulatory DCs induce $CD4^+$ $CD25^+$ human immunoregulatory T cells.

[0076] **FIG. 3C** shows that human immunoregulatory DCs induce $CD4^+$ $CD25^+$ human immunoregulatory T cells.

[0077] **FIG. 4A** shows that human immunoregulatory DCs induce $CD8^+$ $CD28^-$ human immunoregulatory T cells.

[0078] **FIG. 4B** shows that human immunoregulatory DCs induce $CD8^+$ $CD28^-$ human immunoregulatory T cells.

[0079] **FIG. 5A** shows that human immunoregulatory DCs suppress graft-versus-host disease after xenogeneic transplantation caused by human T cells.

[0080] **FIG. 5B** shows that human immunoregulatory DCs suppress graft-versus-host disease after xenogeneic transplantation caused by human T cells.

[0081] **FIG. 6A** shows that murine immunoregulatory DCs suppress human xenogeneic T cell responses.

[0082] **FIG. 6B** shows that murine immunoregulatory DCs suppress human xenogeneic T cell responses.

[0083] **FIG. 6C** shows that murine immunoregulatory DCs suppress human xenogeneic T cell responses.

[0084] **FIG. 6D** shows that murine immunoregulatory DCs suppress human xenogeneic T cell responses.

[0085] **FIG. 6E** shows that murine immunoregulatory DCs suppress human xenogeneic T cell responses.

[0086] **FIG. 7A** shows phenotypes of murine immunoregulatory DCs and also shows that murine immunoregulatory DCs induce antigen-specific anergy to murine T cells and suppress reactivation of activated T cells.

[0087] **FIG. 7B** shows phenotypes of murine immunoregulatory DCs and also shows that murine immunoregulatory DCs induce antigen-specific anergy to murine T cells and suppress reactivation of activated T cells

[0088] **FIG. 7C** shows phenotypes of murine immunoregulatory DCs and also shows that murine immunoregulatory DCs induce antigen-specific anergy to murine T cells and suppress reactivation of activated T cells.

[0089] **FIG. 7D** shows phenotypes of murine immunoregulatory DCs and also shows that murine immunoregulatory DCs induce antigen-specific anergy to murine T cells and suppress reactivation of activated T cells.

[0090] **FIG. 7E** shows phenotypes of murine immunoregulatory DCs and also shows that murine immunoregulatory DCs induce antigen-specific anergy to murine T cells and suppress reactivation of activated T cells.

[0091] **FIG. 7F** shows phenotypes of murine immunoregulatory DCs and also shows that murine immunoregulatory DCs induce antigen-specific anergy to murine T cells and suppress reactivation of activated T cells.

[0092] **FIG. 7G** shows phenotypes of murine immunoregulatory DCs and also shows that murine immunoregulatory DCs induce antigen-specific anergy to murine T cells and suppress reactivation of activated T cells.

[0093] **FIG. 7H** shows phenotypes of murine immunoregulatory DCs and also shows that murine immunoregulatory DCs induce antigen-specific anergy to murine T cells and suppress reactivation of activated T cells.

[0094] **FIG. 8A** shows therapeutic effects of murine immunoregulatory DCs on murine acute graft-versus-host disease after allogeneic transplantation.

[0095] **FIG. 8B** shows therapeutic effects of murine immunoregulatory DCs on murine acute graft-versus-host disease after allogeneic transplantation.

[0096] **FIG. 8C** shows therapeutic effects of murine immunoregulatory DCs on murine acute graft-versus-host disease after allogeneic transplantation.

[0097] **FIG. 5D** shows therapeutic effects of murine immunoregulatory DCs on murine acute graft-versus-host disease after allogeneic transplantation.

[0098] **FIG. 9A** shows the effects of murine immunoregtulatory DCs on immune responses of allogeneic marrow graft recipients and the half-lives after administration of DCs in living mice.

[0099] **FIG. 9B** shows the effects of murine irnmunoregulatory DCs on immune responses of allogeneic marrow graft recipients and the half-lives after administration of DCs in living mice.

[0100] **FIG. 9C** shows the effects of murine immunoregulatory DCs on immune responses of allogeneic marrow graft recipients and the half-lives after administration of DCs in living mice.

[0101] **FIG. 9D** shows the effects of murine immunoregulatory DCs on immune responses of allogeneic marrow graft recipients and the half-lives after administration of DCs in living mice.

[0102] **FIG. 9E** shows the effects of murine immunoregulatory DCs on immune responses of allogeneic marrow graft recipients and the half-lives after administration of DCs in living mice.

[0103] **FIG. 10A** shows the association of murine immunoregulatory T cells with therapeutic effects of murine immunoregulatory DCs for murine acute graft-versus-host disease after allogeneic transplantation.

[0104] **FIG. 10B** shows the association of murine immunoregulatory T cells with therapeutic effects of murine immunoregulatory DCs for murine acute graft-versus-host disease after allogeneic transplantation.

[0105] **FIG. 10C** shows the association of murine immunoregulatory T cells with therapeutic effects of murine immunoregulatory DCs for murine acute graft-versus-host disease after allogeneic transplantation.

[0106] **FIG. 10D** shows the association of murine immunoregulatory T cells with therapeutic effects of murine immunoregulatory DCs for murine acute graft-versus-host disease after allogeneic transplantation.

[0107] **FIG. 10E** shows the association of murine immunoregulatory T cells with therapeutic effects of murine immunoregulatory DCs for murine acute graft-versus-host disease after allogeneic transplantation.

[0108] **FIG. 10F** shows the association of murine immunoregulatory T cells with therapeutic effects of murine immunoregulatory DCs for murine acute graft-versus-host disease after allogeneic transplantation.

[0109] **FIG. 10G** shows the association of murine immunoregulatory T cells with therapeutic effects of murine immunoregulatory DCs for murine acute graft-versus-host disease after allogeneic transplantation.

[0110] **FIG. 10H** shows the association of murine immunoregulatory T cells with therapeutic effects of murine immunoregulatory DCs for murine acute graft-versus-host disease after allogeneic transplantation.

[0111] **FIG. 10I** shows the association of murine immunoregulatory T cells with therapeutic effects of murine immunoregulatory DCs for murine acute graft-versus-host disease after allogeneic transplantation.

[0112] **FIG. 10J** shows the association of murine immunoregulatory T cells with therapeutic effects of murine immunoregulatory DCs for murine acute graft-versus-host disease after allogeneic transplantation.

[0113] **FIG. 11** shows phenotypes of cells induced in vitro by stimulating $CD4^+$ $CD25^-$T cells with murine immunoregulatory DCs.

[0114] **FIG. 12A** shows that murine immunoregulatory DCs suppress murine acute graft-versus-host disease after allogeneic transplantation while maintaining their graft-versus-leukemia effects.

[0115] **FIG. 12B** shows that murine immunoregulatory DCs suppress murine acute graft-versus-host disease after allogeneic transplantation while maintaining their graft-versus-leukemia effects.

[0116] **FIG. 12C** shows that murine immunoregulatory DCs suppress murine acute graft-versus-host disease after allogeneic transplantation while maintaining their graft-versus-leukemia effects.

[0117] **FIG. 13A** shows that murine immunoregulatory DCs suppress murine type II collagen-induced arthritis.

[0118] **FIG. 13B** shows that murine immunoregulatory DCs suppress murine type II collagen-induced arthritis.

[0119] **FIG. 14** shows the effects of the administration of immunoregulatory DCs on EAE.

[0120] **FIG. 15** shows DTH responses.

BEST MODES FOR CARRYING OUT THE INVENTION

[0121] Examples that describe specific embodiments and effects of the present invention are provided below, although the technical scope of the present invention is not limited to these examples.

A: EXAMPLES EMPLOYING HUMAN IMMUNOREGULATORY DCs

[0122] Three types of modified human DCs, i.e., IL-10-induced DCs, TGF-β1-induced DCs, and IL-10/TGF-β1-induced DCs, were prepared. Based on the results attained in Example 2, IL-10/TGF-μ1-induced DCs exhibiting the most potent capacity of regulating T-cell functions were determined to be human immunoregulatory DCs.

Example 1

Phenotypes of modified human DCs

[0123] Human DCs were prepared in the following manner. Human peripheral blood-derived mononuclear cells were allowed to adhere to a dish for cell culture (Becton Dickinson) for 2 hours, and monocytes were obtained as adherent cells (>90% $CD14^+$ cells). These monocytes were cultured in the presence of human GM-CSF (50 ng/ml, PeproTech) and human IL-4 (50 ng/ml, PeproTech) for 7 days, nonadherent cells were recovered, and negative selection was carried out using an anti-CD2 monoclonal antibody (Dynal) and an anti-CD19 monoclonal antibody (Dynal) to which magnetic beads had been coupled to remove contaminating T cells, NK cells, and B cells. Cells remaining after the removal were determined to be immature normal DCs. Similarly, modified human DCs were prepared by culturing monocytes with human IL-10 (50 ng/ml, PeproTech) alone (IL-10-induced DC), human TGF-β1 (50 ng/ml, PeproTech) alone (TGF-β1-induced DC), or human IL-10 (50 ng/ml, PeproTech) in combination with human TGF-β1 (IL-10/TGF-β1-induced DC) in the presence of human GM-CSF (50 ng/ml) and human IL-4 (50 ng/ml) for 7 days, and then removing contaminating T cells, NK cells, and B cells as with the case of the aforementioned immature DCs. Human IL-10-treated immature DCs were obtained by allowing human IL-10 (50 ng/ml, PeproTech) to act on immature DCs similar to the aforementioned DCs for 3 days. Mature DCs were prepared in the following manner. In order to keep the cells from becoming contaminated with cytokines, the aforementioned cells were washed three times with PBS, cultured in the presence of human TNF-α (50 ng/ml, PeproTech) for an additional 3 days, and the resultants were determined to be mature DCs. The obtained DCs were analyzed using the FACScan flow cytometer (Becton Dickinson), 95% or more thereof were found to be HLA-DR-expressing, cells, and contamination with T cells, B cells, NK cells, or monocytes/macrophages was not more than 0.1%. Phenotypes of the thus prepared immature/mature human DCs, immature/mature human IL-10-induced DCs, immature/mature human TGF-β1-induced DCs, and immature/mature human IL-10/TGF-β1-induced DCs were analyzed by a flow cytometer. The results yielded representative data for 10 separate experiments. **FIG. 1A** shows the results of staining using an anti-CD1a antibody, an anti-CD14 antibody, an anti-CD11c antibody, an anti-CD83 antibody, an anti-E-cad antibody, and isotype controls thereof (BD PharMingen). **FIG. 1B** shows the results of staining using an anti-CD40 antibody, an anti-CD80 antibody, an anti-CD86 antibody, an anti-HLA/A/B/C antibody, an anti-HLA-DR antibody, and isotype controls thereof (BD PharMingen). Numerical values presented in the upper right of the drawing independently represent mean fluorescence intensity when stained with an antibody. These values indicate that DC markers for all cells, i.e., CD1a and CD11c, were expressed in the case of immature DCs, although the Langerhans cell marker, E,-cadherin (E-cad), was not expressed in the IL-10-induced cells as with the case of normal DCs. In the case of IL-10-induced DCs, expression of CD14, which is not observed in other DCs, was observed. Concerning HLA-A/B/C and HLA-DR, a moderate level of expression was

observed in IL-10-induced DCs, TGF-β1-induced DCs, and IL-10/TGF-β1-induced DCs, although this expression level was somewhat lower than that in immature normal DCs. Expression levels of CD40, CD80, and CD86 were very low. Concerning DCs that were allowed to mature with the aid of TNF-α, i.e., IL-10/TGF-β1-induced DCs, the expression level of the DC activation marker CD83 was elevated. However, expression levels of the DC markers CD1a and CD11c and the Langerhans cell marker E-cad were lowered. Expression levels of CD83, CD40, CD80, and CD86 were lower in IL-10-induced DCs, TGF-β1-induced DCs, and IL-10/TGF-β1-induced DCs compared with those in mature normal DCs. Expression levels thereof were significantly low particularly in IL-10/TGF-β1-induced DCs. Concerning expressions of HLA and co-stimulatory factors, the ratios of cells to be expressed obtained in 10 separate experiments are presented in terms of mean±SD in **FIG. 1C** and in terms of MFI±SD in **FIG. 1D**.

Example 2

Among Modified Human DCs. IL-10/TGF-β1-Induced DCs Function as Immunoregulatory DCs to Induce Anitgen-Specific Anergy to T Cells and Suppress Reactivation of Actived T Cells

[0124] Whether or not modified DCs would induce anergy to allogeneic $CD4^+$ T cells was examined. T cells were isolated from human peripheral blood using a negative selection kit (Dynal), and naive $CD4^+$ T cells (10^5 cells), which had been isolated as $CD8^-CD45RO^-$cells using an anti-CD8 antibody and an anti-CD45RO antibody (BD PharMingen), were cultured with allogeneic DCs or allogeneic modified DCs (10^3 to 10^4 cells) for 5 days to conduct cell growth assay. In another experiment, naive $CD4^+$ T cells (5×10^6 cells) were cultured with X-ray (15 Gy)-irradiated allogeneic DCs or allogeneic modified DCs (4×10^4 to 5×10^5 cells) for 3 days, and negative selection was carried out using an anti-CD11c antibody and a magnetic-beads-coupled goat anti-mouse IgG antibody to recover $CD4^+$ cells. The recovered $CD4^+$ cells (10^5 cells) were subjected to the second culture together with allogeneic normal mature human DCs (10^4 cells) derived from the same donor ass in the case of the first stimulation or allogeneic normal mature human DCs (10^4 cells) derived from a donor different from that of the case of the first stimulation in the presence or absence of IL-2. Cell growth assay was carried out on the fifth day. In the cell growth assay, cells were pulsed with $[^3H]$thymidine for 18 hours, and incorporation of $[^3]$thymidine into cells was employed as an indicator. As shown in **FIG. 2A**, assay was carried out using IL-10-induced DC, TGF-β1-induced DC, IL-10/TGF-β1-induced DC, or IL-10-treated DC. In the case of immature DCs, the capacity of modified DCs to activate allogeneic $CD4^+$ T cells at the time of primary stimulation was uniformly low, and that of immature IL-10/TGF-β1-induced DCs was the lowest. When mature DCs were used, however, the capacity of IL-10/TGF-β1-induced DCs to activate allogeneic $CD4^+$ T cells at the time of primary stimulation was significantly lower than that of other mature DCs. When primarily stimulated with each of the DCs and then secondarily stimulated with mature normal DCs, growth was suppressed only in immature IL-10/TGF-β1-induced DCs or mature IL-10/TGF-β1-induced DCs. In the case of IL-10-treated immature DCs, potent suppressing activities were observed in both experiments for primary and secondary stimulations, although this suppressing activity was not as potent as that of IL-10/TGF-β1-induced DCs In FIG, **2B**, suppressing activity tended to cease with the addition of IL-2 at the time of secondary stimulation after stimulation with IL-10/TGF-β1-induced DCs. When mature normal DCs derived from an unrelated donor were used for secondary stimulation, the level of suppression was insignificant. This indicates that IL-10/TGF-β1-induced DCs induce anergy to naive $CD4^+$ cells in an antigen-specific manner. Similar results were observed when all of the $CD4^+$ T cells were used as responders, and the level of suppression was more potent than that of the IL-10-treated immature DCs (**FIG. 2C**). The suppression effects attained at the time of secondary stimulation with mature normal DCs were exhibited in a manner dependent on the dosage of the IL-10/TGF-β1-induced DCs added at the time of primary stimulation (**FIG. 2D**). Changes in cell growth after secondary stimulation were inspected with the elapse of lime, and cell growth suppressing effects were maintained for at least two weeks (**FIG. 2E**). Further, the activity of IL-10/TGF-β1-induced DCs upon naive $CD4^+$ cells that had been activated by mature allogeneic DCs was examined. This revealed that IL-10/TGF-β1-induced DCs suppressed the cell growth in a dose-dependent manner (**FIG. 2F**). Subsequently, the activity of IL-10/TGF-β1-induced DCs upon the cytotoxicity of antigen-specific $CD8^+$ T cells was examined (**FIG. 2G**). T cells were isolated from human peripheral blood using a negative selection kit (Dynal), and naive $CD8^+$ T cells were isolated as $CD4^-$ and $CD45RO^-$ cells using an anti-CD4 antibody and an anti-CD45RO antibody (BD PharMingen). Antigen-specific $CD8^+$ T cells were obtained by culturing X-ray (15 Gy)-irradiated allogeneic fibroblasts (donor #1) and PBMC for 2 weeks (100 U/ml of IL-2 added) and subjecting $CD8^+$ T cells to positive selection. The antigen-specific $CD8^+$ cells were cultured in the presence or absence of allogeneic DCs (donor #1 or #2) for 3 days. Cytotoxicity assay was carried out by culturing the $CD8^+$ T cells (5×10^5 cells) with allogeneic fibroblasts labeled with $Na_2{}^{51}CrO_4$ (100 μCi/10^6 cells, NEN™ Life Science Product, Boston, Mass.) (donor #1 or #2) for 4 hours and assaying the radioactivity of the culture supernatant. As a result, $CD8^+$ T cells were found to exhibit cytotoxicity in a manner specific to the allogeneic fibroblasts (donor #1) used for stimulation, i.e., in an antigen-specific manner, In this case, cytotoxicity was enhanced when stimulation took place with mature normal DCs instead of immature normal DCs. In contrast, IL-10/TGF-β1-induced DCs suppressed cytotoxicity in a dose-dependent manner. When unrelated donor-derived DCs were used, cytotoxicity was not substantially affected. Specifically, immunoregulatory activity caused by the IL-10/TGF-β1-induced DCs was considered to be specific. Accordingly, IL-10/TGF-β1-induced DCs were found to be capable of regulating activities of all effector T cells. The IL-10/TGF-β1-induced DCs are hereafter referred to as "immunoregulatory DCs."

Example 3

Human Immunoregulatory DCs Inudce $CD4^+$ $CD25^+$ Immunoregulatory T Cells

[0125] Human naive $CD4^+$ T cells (5×10^6 cells) isolated in a manner equivalent to that of Example 2 were cultured

together with allogeneic DCs or allogeneic immunoregulatory DCs (5×10^5 cells) for 5 days. The obtained T cells were analyzed for cell surface antigens and intracellular cytokines using FACS. Intracellular cytokine production was analyzed in the following manner. Cells were stimulated with an anti-human CD3 antibody immobilized on a plate (10 μg/ml, BD PharMingen) and with a solubilized anti-human CD28 antibody (10 μg/ml, BD PharMingen) for 6 hours. The resulting cells were permeated, immobilized, and then stained with anti-human IL-2, IL-4, IL-10, and interferon (IFN)-γ (BD PharMingen) for analysis using FACS. The results represent one typical data set attained in 5 separate experiments. When allogeneic normal DCs were used, $CD4^+$ $CD25^+$ cells and $CD4^+$ $CD154^+$ cells were induced. In contrast, $CD4^+$ $CD25^+$ cells and $CD4^+$ $CD152^+$ cells were induced when allogeneic immunoregulatory DCs were used (**FIG. 3A**). Concerning intracellular cytokine production, IFN-γ- and IL-2-producing cells increased when stimulated with allogeneic normal DCs whereas IL-10-producing cells increased when stimulated with allogeneic immunoregulatory DCs (**FIG. 3B**). Further, functions of $CD4^+$ $CD25^+$ T cells induced with the aid of allogeneic immunoregulatory DCs were analyzed (**FIG. 3C**). The method was as described below. Human naive $CD4^+$ T cells (5×10^6 cells) were cultured together with X-ray (15 Gy)-irradiated allogeneic normal mature DCs (5×10^5 cells) for 3 days, negative selection was carried out using an anti-CD11 c antibody and a magnetic-beads-coupled goat anti-mouse IgG antibody, and antigen-stimulated $CD4^+$ cells were obtained. Human $CD4^+$ $CD25^+$ T cells were isolated by culturing naive $CD4^+$ T cells (5×10^6 cells) with allogeneic immature immunoregulatory DCs (5×10^5 cells) for 5 days and using an anti-CD25 antibody (BD PharMingen) and a magnetic-beads-coupled goat anti-mouse IgG antibody. As a result of FACS analysis, purity was found to be 95% or higher. The obtained antigen-stimulated $CD4^+$ cells were mixed with a different amount of $CD4^+$ $CD25^+$ T cells, the resultant was cultured with allogeneic mature normal DCs (10^4 cells) for an additional 5 days, and cell growth was then assayed. While the antigen-stimulated $CD4^+$ cells alone responded to allogeneic mature normal DCs and abundantly grew, $CD4^+$ $CD25^+$ T cells alone did not substantially responded thereto. When $CD4^+$ $CD25^+$ T cells were cultured together with antigen-stimulated $CD4^+$ cells, they suppressed the growth of stimulated $CD4^+$ cells in a dose-dependent manner. However, the suppression effects were not dissolved even though the number of $CD4^-$ $CD25^+$ T cells was maintained at a constant level while the number of antigen-stimulated $CD4^+$ cells was increased. This indicates that cell growth is not merely suppressed by competitive inhibition against allogeneic antigens. This suppression effect disappears when the $CD4^+$ $CD25^+$ T cells are separated from the stimulated $CD4^+$ cells in a transwell, and the suppression activity partially disappears with the addition of IL-2. An IL-10- or TGF-β-neutralizing antibody did not affect the suppression activity (data is not shown) In the case of $CD4^+$ $CD25^+$ T cells (donor A, induced by immunoregulatory DCs of allogeneic donor B), suppression of the activation of naive $CD4^+$ T cells (donor A) by allogeneic mature normal DCs (donor B) is twice as potent as suppression of the activation of naive $CD4^+$ T cells (donor A) by allogeneic mature normal DCs (donor C). This indicates that the suppression activity by $CD4^+$ $CD25^+$ T cells can be antigen-specific or non-specific (data is not shown). Accordingly, immunoregulatory DCs effectively induce $CD4^+$ $CD25^+$ immunoregulatory T cells.

Example 4

Human Immunoregulatory DCs Induce $CD8^+$ $CD28^-$ Immunoregulatory T Cells

[**0126**] Human naive $CD8^+$ T cells (5×10^6 cells) isolated in a manner similar to that of Example 2 were cultured together with X-ray (15 Gy)-irradiated allogeneic normal DCs or allogeneic immunoregulatory DCs (5×10^5 cells) for 5 days, and negative selection was earned out using an anti-CD11c antibody and a magnetic-beads-coupled goat anti-mouse IgG antibody to recover $CD8^+$ T cells. These cells were subjected to analysis of cell surface antigens (left in **FIG. 4A**) and inspection of intracellular cytokine production. Cell surface antigens were analyzed in accordance with Example 1, and intracellular cytokines were assayed in the following manner. Cells stimulated with PMA (20 ng/ml, Sigma) and Ca^{2+} ionophore A23187 (500 ng/ml, Sigma) for 6 hours were permeated, immobilized, stained with anti-human IL-10 and IFN-γ, and then analyzed using FACS. The analysis revealed that $CD8^+$ $CD28^+$ cells were induced when naive CDs^{8+} T cells were cultured together with allogeneic normal DCs whereas $CD8^+$ $CD28^-$ cells were induced when naive $CD8^+$ T cells were cultured together with allogeneic immunoregulatory DCs (**FIG. 4A**). When intracellular cytokines were inspected, the number of INF-γ-producing cells increased when naive $CD8^+$ T cells were cultured together with allogeneic normal DCs. In contrast, when naive $CD8^+$ T cells were cultured together with allogeneic immunoregulatory DCs, the number of IL-10-producing cells increased (**FIG. 4A**). Further, functions of $CD8^+$ $CD28^+$ cells obtained by culturing with allogeneic mature DCs in the aforementioned manner and those of $CD8^+$ $CD28^-$ cells obtained by culturing with allogeneic immature immunoregulatory DCs were analyzed $CD8^+$ $CD28^+$ T cells or $CD8^+$ $CD28^-$ T cells (10^4 to 10^5 cells) were cultured together with X-ray-irradiated allogeneic mature normal DCs (10^4 cells) and antigen-stimulated $CD4^+$ T cells (10^5 cells) prepared in the same manner as in Example 3, and cell growth assay was carried out 5 days thereafter. In a transwell experiment utilizing a 24-well plate, X-ray-irradiated allogeneic mature normal DCs (10^5 cells) were added to $CD8^+$ $CD28^+$ T cells or $CD8^+$ $CD28^-$ T cells (10^6 cells), antigen-stimulated $CD4^+$ T cells (10^5 cells) and X-ray-irradiated allogeneic normal mature DCs (10^5 cells) were directly added thereto or partitioned in separate transwell chambers, and the resultant was cultured for 5 days. DCs were removed in the same manner as in Example 2 five days thereafter, T cells (10^5 cells) were transferred to a 96-well plate, and cell growth assay was carried out. When $CD8^+$ $CD28^+$ T cells were cultured together with allogeneic mature normal DCs or when antigen-stimulated $CD4^+$ T cells were cultured together with allogeneic mature normal DCs, $CD4^+$ or $CD8^+$ $CD28^+$ T cells was found to have been grown. In contrast, $CD8^+$ $CD28^-$ T cells did not substantially grow when $CD8^+$ $CD28^-$ T cells were cultured together with allogeneic murine normal mature DCs (**FIG. 4B**). Further, $CD8^+$ $CD28^-$ T cells suppressed the growth of $CD4^+$ T cells caused by allogeneic mature normal DCs in a dose-dependent manner (**FIG. 4B**). Transwell experiment revealed that contact between $CD4^+$ T cells and $CD8^+$ $CD28^-$ cells was necessary for this suppression activity (**FIG. 4B**). This

demonstrates that immunoregulatory DCs induce $CD8^+$ $CD28^-$ immunoregulatory T cells from naive $CD8^+$ T cells.

Example 5

Human Immunoregulatory DCs Suppress Graft-Versus-Host Disease after Xenogeneic Transplantation Caused by Human T Cells

[0127] Effects of human immunoregulatory DCs in models of graft-versus-host disease (GvHD) after xenogeneic transplantation (the process is described in Example 7) were examined. Human immunoregulatory DCs were induced in the same manner as in Example 1. Normal immature human DCs or immature human immunoregulatory DCs were pulsed with necrotized spleen cells of BALB/c mice (10^5 cells) for 24 hours, culture was carried out in the presence of TNF-α (50 ng/ml) for 3 days, and cultured cells were then allowed to mature. Necrotized cells were prepared by subjecting cells to a freeze/thaw cycle four times. Xenogeneic GvHD responses were induced in the same manner as described in Example 7, and the aforementioned cells (4×10^6 cells) were administered through caudal veins 2 days after the induction. As a result, the mice died due to administration of normal mature human DCs significantly earlier than the control group, and administration of mature human immunoregulatory DCs significantly prolonged their survival (**FIG. 5A**). In the same manner as in Example 6, human T cells were separated from spleen cells 10 days after administration, and reactivity with murine normal mature DCs was assayed. As a result, human T cells derived from mice to which normal mature human DCs had been administered exhibited significantly higher reactivity compared with that of the control group, and human immunoregulatory T cells derived from mice to which murine normal mature DCs had been administered exhibited significantly lower reactivity compared with that of the control group (**FIG. 5B**).

B: EXAMPLES EMPLOYING MURINE IMMUNOREGULATORY DCS

Example 6

Method of Preparing Murine DCs and Murine T Cells, Method of Stimulating T Cells in vivo, and Method of Experimentation Utilizing T Cells

[0128] Murine normal mature DCs (mDCs) were prepared by culturing bone marrow cells obtained from BALB/c, C57BL/6, DBA/1, or CBA/1 mice in a plastic culture dish in the presence of recombinant murine GM-CSF (20 ng/ml, PeproTech, London, England) for 6 days, and conducting further culture in the presence of LPS (1 μg/ml, Sigma, St. Louis, Mo.) for 2 days. Murine immunoregulatory DCs (rDCs) were prepared by culturing murine bone marrow cells obtained from mice of the same strain as the murine normal mature DCs in a plastic culture dish in the presence of recombinant murine GM-CSF (20 ng/ml, PeproTech, London, England), recombinant murine IL-10 (20 ng/ml, PeproTech, London, England), and recombinant human TGF-β1 (20 ng/ml, PeproTech, London, England) for 6 days, and then conducting further culture in the presence of LPS (1 μg/ml, Sigma, St. Louis, Mo.) for 2 days. Dihydroxyvitamin D_3-conditioned DCs were prepared by culturing murine bone marrow cells obtained from mice of the same strain as the murine normal mature DCs in a culture dish in the presence of recombinant murine GM-CSF (20 ng/ml, PeproTech, London, England) and dihydroxyvitamin D_3 (10 nM, Sigma, St. Louis, Mo.) for 6 days, and then conducting further culture in the presence of LPS (1 μg/ml, Sigma, St. Louis, Mo.) for 2 days.

[0129] T cell fractions were prepared in the following manner. Specifically, spleen mononuclear cells fractions of normal mice ($H\text{-}2^d$, $H\text{-}2^b$, or $H2^k$) were suspended in PBS, rat antibodies against Ly-76, B220, Ly-6G, and I-A/I-E (BD PharMingen, San Diego, Calif.) were added, incubation was carried out at 4° C. for 30 minutes, cells were washed with PBS, sheep anti-rat IgG mAb-conjugated immunomagnetic beads (Dynal, Oslo, Norway) were added, incubation was carried out again at 4° C. for 30 minutes, cells were washed with PBS, and T cell fractions were obtained by negative selection. A rat anti-CD8 or CD4 antibody (BD PharMingen, San Diego, Calif.) and sheep anti-rat IgG mAb-conjugated immunomagnetic beads were added to aforementioned T cell fractions in the same manner described above, and $CD4^+$ T cell fractions or $CD8^+$ T cell fractions were obtained by negative selection. As a result of analysis using a flow cytometer FACScan (Becton Dickinson, Mountain View, Calif.), purities of these T cell fractions were all 97% or higher.

[0130] Bone marrow cells (1.5×10^7 cells/mouse) and spleen mononuclear cells (1.5×10^7 cells/mouse) (BMS) derived from allogeneic donor mice were administered intravenously to recipient mice to which lethal doses of X-rays had been applied (10 Gy/mouse) and then transplanted. Combinations of a recipient mouse with a donor mouse were: 1) BALB/c($H\text{-}2^d$) with C57BL/6($H\text{-}2^b$); 2) C57BL/6($H2^b$) with BALB/c($H\text{-}2^d$); or 3) DBA/l($H\text{-}2^q$) with BALB/c($H\text{-}2^d$). Spleen mononuclear cells were recovered 5 days after transplantation, recipient cells were removed by negative selection using an anti-recipient type I-K mouse antibody and anti-mouse IgG microbeads to prepare donor-derived spleen mononuclear cell fractions. The yield of this fraction was 2×10^7 cells/mouse or lower, and the content of donor type $I\text{-}K^+$ cell was 95% or higher. $CD4^+$ T cell fractions and $CD8^+$ T cell fractions were prepared in the same manner as in Example 6, and the yield thereof was 4×10^6 cells/mouse.

[0131] Similarly, donor-derived $CD4^+$ T cells and $CD8^+$ T cells were recovered from the spleen mononuclear cell fraction of recipient mice to which allogeneic transplantation had been applied and murine normal mature DCs or murine immunoregulatory DCs bad been then administered. The yield thereof was 1×10^7 or 3×10^7 cells/mouse or lower. The recovered cells were cultured in the presence of recombinant murine IL-2 (10 ng/ml) for 3 days and then used in the assay.

Example 7

Murine Immunoregulatory DCs Suppress Human Xenogeneic T Cell Responses

[0132] Murine normal mature DCs and murine immunoregulatory DCs were prepared in the same manner as in Example 6. Phenotypes were analyzed using a flow cytometer. An anti-CD11c antibody, an anti-CD40 antibody, an anti-CD80 antibody, an anti-CD86 antibody, an anti-$I\text{-}K^d$

antibody, an anti-I-A/I-E antibody, and isotype controls thereof (BD PharMingen) were used for staining. Numerical values presented in the upper right of **FIG. 6A** independently represent mean fluorescence intensity when stained with an antibody. As a result, significant difference was not observed in the expression of I-A/I-K molecules in the case of murine modified DCs compared with the case of murine normal mature DCs, although expression levels of CD40, CD80, and CD86 (costimulating molecules) were lower in murine modified DCs.

[0133] The capacity for activating human T cells was examined. This demonstrated that murine immunoregulatory DCs had lower capacity for activating human T cells compared with murine normal mature DCs (data is not shown). While human T cells that had been activated by murine normal mature DCs were reactivated by murine normal mature DCs, human T cells that had been activated by murine immunoregulatory DCs were not reactivated by murine normal mature DCs (data is not shown).

[0134] Functions of murine immunoregulatory DCs in xenogeneic GvHD were further examined. Xenogeneic GvHD was induced in the following manner. PBL (5×10^7 cells) were cultured together with X-ray (15 Gy)-irradiated murine normal mature DCs or murine immunoregulatory DCs (H-2^d) (5×10^6 cells) in the presence or absence of human IL-2 (100 U/ml) for 3 days, negative selection for human T cells was carried out using an anti-l-K^d antibody (BD PharMingen) and goat anti-mouse IgG antibody-conjugated immunomagnetic beads, culture was further carried out in the presence of human IL-2 (10 U/ml) for 3 days, and human T cells stimulated with xenoantigens were obtained. Anti-asialo GM1 antiserum (20 μl, 10 mg/ml, Wako Pure Chemical Industries, Ltd.) was administered to C.B.-17-scid recipient mice (H-2^d) one day before cell transplantation, and a sublethal dose of X-rays (5 Gy) was applied on the day of cell transplantation. Subsequently, human T cells stimulated with xenoantigens or unstimulated human T cells (4×10^7 cells) were administered to mice intraveneously. Two days after the administration of human T cells, murine normal mature DCs (4×10^6 cells) or murine immunoregulatory DCs (4×10^6 cells) obtained in the same manner as in Example 6 were administered. The group to which murine immunoregulatory DCs and human IL-2 (10^4 U) were to be simultaneously administered on days 3, 5, and 7 was also provided. As a result, mice (the control group) to which human T cells had been transplanted died within 24 days after the cell transplantation due to xenogeneic GvHD responses. When human T cells stimulated with murine normal mature DCs were transplanted to mice, they died significantly earlier than those in the control group ($P<0.01$). In contrast, mice survived longer when human T cells stimulated with murine immunoregulatory DCs had been transplanted. When human T cells stimulated with murine immunoregulatory DCs had been transplanted to mice in the presence of IL-2 (100 U/ml), however, the survival of mice was curtailed (**FIG. 6B**). Ten days after the transplantation, human T cells were recovered from spleen cells of the recipient mice, and their reactivity with murine normal mature DCs was examined in the following manner. Mononuclear cells were recovered from spleen cells using a HISTOPAQUE-1080 (Sigma), negative selection was carried out using an anti-I-K^b antibody and goat anti-mouse IgG antibody-conjugated immunomagnetic beads to prepare human T cells, and the resultant was cultured in the presence of human IL-2 (10 U/ml) for 3 days. These human T cells (10^5 cells) were cultured together with normal mature murine DCs (10^3 to 5×10^4 cells) for 5 days, and cell growth assay was carried out. The assay revealed that human T cells derived from mice to which human T cells stimulated with murine normal mature DCs had been transplanted had higher reactivity with murine normal mature DCs than the control group to which human T cells only had been transplanted (**FIG. 6C**) In contrast, human T cells derived from mice to which human T cells stimulated with murine immunoregulatory DCs had been transplanted exhibited low reactivity with murine normal mature DCs (**FIG. 6C**). Survival of the xenogeneic GvHD models was prolonged when murine immunoregulatory DCs had been administered after human T cell transplantation. This effect of prolonged survival was abrogated by IL-2 administration (**FIG. 6D**).

[0135] In order to analyze therapeutic effects of murine immunoregulatory DCs on acute GvHD caused by allogenic bone marrow transplantation utilizing SCID mice, the influence of a single administration of murine immunoregulatory DCs (H-2^d) on lethal GvHD, which developed in the recipient mice (H-2^d) to which allogeneic bone marrow cells and spleen mononuclear cells (H-2^b) had been transplanted, was inspected. The SCID mice (H-2^d) were systemically irradiated with a lethal dose of radioactive rays (10 Gy/mouse), and C57BL/6 mice-derived bone marrow cells (H-2^b, 2×10^7 cells/mouse) and G57BL/6 mice-derived spleen mononuclear cells (H-2^b, 2×10^7 cells/mouse) prepared in the manner described above were administered. Two days later, a group to which murine normal mature DCs (4×10^6 cells/mouse) or murine immunoregulatory DCs (4×10^6 cells/mouse) prepared in the manner described above were to be administered and a group to which DCs were not to be administered were provided, and the survival (%) of these groups thereafter was observed. All samples in the group to which DCs had not been administered died within 18 days after the transplantation, and all samples in the group to which murine normal mature DCs had been administered died earlier than those in the former group (within 12 days). However, all samples in the group to which murine immunoregulatory DCs had been administered were still alive 120 days after the transplantation. This demonstrates that murine immunoregulatory DCs have therapeutic effects on acute graft-versus-host disease that is developed after allogeneic bone marrow transplantation using SCID mice (**FIG. 6E**).

Example 8

Comparison of Cell Surface Molecule Expression of Murine Immunoregulatory DCs with that of Murine Normal Mature DCs and functions of Murine Immunoregulatory DCs

[0136] Murine normal mature DCs or murine immunoregulatory DCs prepared in accordance with the method described in Example 6 were washed with PBS, fluorescein-conjugated antibodies specific to DC markers (CD11c), costimulating molecules (CD40, CD80, and CD86), or MHC molecules (I-K^d and I-A/I-E) were added, and incubation was then carried out under ice cooling for 30 minutes. After washing with PBS, the expression of each molecule was inspected using a flow cytometer (Becton Dickinson). In murine normal mature DCs (H-2^d), CD11c, CD40, CD80, CD86, I-K^d, and I-A/I-E were expressed with high intensity

and high frequency. In murine immunoregulatory DCs, CD11c and MHC molecules were expressed with high frequency, although expression levels of CD40, CD80, and CD86 were significantly low (Table 1 and **FIG. 7A**). Expression patterns of cell surface molecules for murine immunoregulatory DCs derived from all the examined mouse strains (H-2^d, H_2^b, and H-2^q) exhibited the same inclination (Table 1).

been incorporated in cells was recovered onto a glass filter from the culture plate using a cell harvester, dehydrated, thoroughly penetrated with aquasol, and packaged in a dedicated-purpose film. The β-dose was measured using a β counter to inspect the activation of T cell growth caused by DCs. This demonstrates that murine normal mature DCs (H-2^d) potently activate allogeneic $CD4^+$ T cells (H-2^b or H-2^k). In contrast, the capacity of murine immunoregulatory

TABLE 1

		Phenotypes and capacity for stimulating allogeneic T cells of immunoregulatory DCs induced from a variety of mouse strains				
		CD40	CD?1c	CD80	CD86	I-K
BALB/c mice (H-2^1) (n = 10)	mDCs	68 ± 8/215 ± 45	58 ± 7/252 ± 33	84 ± 8/440 ± 53	77 ± 9/994 ± 74	86 ± 10/640 ± 75
	rDCs	4 ± 2/14 ± 3	60 ± 5/243 ± 41	5 ± 3/23 ± 11	10 ± 3/36 ± 12	48 ± 10/359 ± 48
	D_1-conditioned DCs	32 ± 5/130 ± 15	47 ± 5/178 ± 24	29 ± 6/111 ± 28	28 ± 7/257 ± 36	42 ± 5/284 ± 43
C57/BL6 mice (H-2?) (n = 10)	mDCs	65 ± 7/204 ± 34	61 ± 5/294 ± 41	80 ± 10/387 ± 49	75 ± 8/979 ± 68	83 ± 12/628 ± 64
	rDCs	3 ± 2/12 ± 5	54 ± 4/236 ± 45	4 ± 2/18 ± 9	8 ± 3/24 ± 7	47 ± 3/385 ± 51
DBA/I mice (H-2?) (n = 10)	mDCs	64 ± 10/198 ± 33	63 ± 8/302 ± 48	76 ± 12/355 ± 42	72 ± 9/945 ± 54	81 ± 13/612 ± 58
	rDCs	3 ± 2/10 ± 4	51 ± 9/244 ± 32	5 ± 3/19 ± 8	7 ± 2/21 ± 6	46 ± 7/344 ± 53
BALB/c mice (H-2?) (n = 10)	rDCs[b]	5 ± 3/18 ± 5	57 ± 7/231 ± 29	6 ± 2/30 ± 9	8 ± 4/42 ± 12	52 ± 12/378 ± 51

			Tcell (H-2?)?DC ratio		
		I-A and/or I-E	10:1	100:1	200:1
BALB/c mice (H-2^1) (n = 10)	mDCs	77 ± 12/2405 ± 324	39547 ± 2414	15474 ± 1252	4321 ± 341
	rDCs	73 ± 11/1162 ± 187	541 ± 121	194 ± 64	154 ± 18
	D_1-conditioned DCs	48 ± 6/687 ± 85	12654 ± 554	3354 ± 654	1145 ± 221
C57/BL6 mice (H-2?) (n = 10)	mDCs	75 ± 14/2302 ± 287	35784 ± 1987	13421 ± 1754	3982 ± 405
	rDCs	74 ± 12/1039 ± 155	334 ± 94	144 ± 34	139 ± 64
DBA/I mice (H-2?) (n = 10)	mDCs	74 ± 11/2159 ± 266	33541 ± 2154	11815 ± 1554	3451 ± 364
	rDCs	71 ± 14/1124 ± 265	297 ± 14	133 ± 14	128 ± 34
BALB/c mice (H-2?) (n = 10)	rDCs[b]	70 ± 8/1243 ± 194	425 ± 84	189 ± 72	121 ± 34

[a]The value of (?H?thymidine incorporation of T cells alone was less than 100 ?.
[b]DCs were obtained from the spleen in rDC (H-2?)-treated recipients (H-2?) of allogeneic BMS (H-2?) on 5 days after transplantation as described in Experimental Procedures.
? indicates text missing or illegible when filed

[0137] Lymphocytes having different histocompatible antigens (antigen-presenting cells and T cells) were subjected to mixed-culture. Thus, the capacity of T cells to activate and to grow alloantigens can be inspected in vitro. The capacities of murine immunoregulatory DCs and murine normal mature DCs to activate allogeneic T cells were inspected in the following manner. More specifically, unprimed or primed $CD4^+$ T cells (2×10^5 cells) were cultured together with X-ray (15Gy) irradiated allogeneic murine normal nature DCs, murine immunoregulatory DCs, or dihydroxyvitamin D_3-conditioned DCs (10^3 to 2×10^5 cells) in the presence or absence of recombinant murine IL-2 at 37° C. in 5% CO_2 in a 96-well culture plate for 3 days. Thereafter, ^{3}H thymidine (Amersham Life Science, Buchinghamshire, UK) was added in an amount of 1 μCi/well, and culture was further conducted at 37° C. in 5% CO_2 in a 96-well culture plate for 16 hours. ^{3}H thymidine that had

DCs to activate allogeneic $CD4^+$ T cells was lower than that of murine normal mature DCs or dihydroxyvitamin D_3-conditioned DCs known as immunotolerance-inducible DCs (Table 1 and **FIG. 7B**). Capacities of murine immunoregulatory DCs derived from all the examined mouse strains (H-2^d, H-2^b, and H-2^q) to activate allogeneic T cells tended to be similar to one another (Table 1).

[0138] Influence of murine immunoregulatory DCs upon $CD4^+$ T cells subjected to allogeneic stimulation in vivo was inspected. Specifically, $CD4^+$ T cells (I-K^{b+} $CD4^+$) obtained from recipient mice subjected to allogeneic bone marrow transplantation were cultured with mature normal DCs derived from allogeneic mice or murine immunoregulatory DCs (H-2^d) in a plastic culture plate in accordance with the method described in Example 6, and activation of $CD4^+$ T cells was inspected. When murine normal mature DCs (H-2^d) were further added to a culture system of I-K^{b+} $CD4^+$

T cells in combination with murine normal mature DCs (H-2^d), the activation of I-K^{b+} $CD4^+$ T cells was slightly enhanced. In contrast, when murine immunoregulatory DCs (H-2^d) were added to the same culture system, activation induced by murine normal mature DCs (H-2^d) was suppressed in accordance with the number of murine immunoregulatory DCs added. In contrast, when murine normal mature DCs prepared from an unrelated mouse strain (H-2^q) were added, I-K^{b+} $CD4^+$ T cells were activated in a more potent manner. When murine immunoregulatory DCs derived from an unrelated mouse strain (H-2^q) were added, no or substantially no influence was imposed upon the activation of I-K^b+$CD4^+$ T cells caused by murine normal mature DCs (H-2^d) (**FIG. 7C**). Accordingly, suppression of $CD4^+$ T cell activation induced by murine immunoregulatory DCs was suggested to be an antigen-specific response. Similar experimental results were obtained with DC-T cell combinations of a strain different from the aforementioned one (**FIG. 7D**).

[**0139**] Influence of murine immunoregulatory DCs on the activity of $CD8^+$ T cells subjected to allogeneic stimulation in vivo was inspected. Specifically, $CD8^+$ T cells (I-K^{b+} $CD8^+$) obtained from recipient mice subjected to allogeneic bone marrow transplantation were cultured with mature normal DCs derived from allogeneic mice or murine immunoregulatory DCs (H-2^d) in a plastic culture plate in accordance with the method described in Example 6, and the activation of $CD8^+$ T cells was inspected. Cytotoxicity of $CD8^+$ T cells stimulated in vivo was inspected in the following manner. Specifically, $CD8^+$ T cells stimulated in vivo and in vitro were mixed with P815 cells, EL4 cells, and Con A-blast cells that had been radioactively labeled with $Na_{51}CrO_4$ (10^4 cells) at various mixing ratios, the resultants were subjected to culture for 4 hours, the culture supernatants were recovered, and the activity of radioactive substances contained therein was assayed. I-K^{b+} $CD8^+$ T cells subjected to allogeneic stimulation in vivo exhibited potent cytotoxicity against the cell strain P815 (H-2^d), which was syngeneic to the stimulation. However, they did not exhibit any activity against the cell strain EL4 (H-2^b) or Con A-blast (H-2^q), which were strains different therefrom (**FIG. 7E**). This indicates that cytotoxicity induced in $CD8^+$ T cells stimulated with H-2^d in vivo is specific to H-2^d. When I-K^{b+} $CD8^+$ T cells subjected to stimulation in vivo were cultured together with murine immunoregulatory DCs (H-2^d), however, their cytotoxicity against P815 was significantly suppressed. When they were cultured together with syngeneic murine immunoregulatory DCs (H-2^b) or the immunoregulatory DCs of unrelated mica (H-2^q), their activity was not substantially affected (**FIG. 7E**). These results indicate that suppression of CTL activity by murine immunoregulatory DCs was an antigen-specific response. Similar results were attained with combinations of different mouse strains (**FIG. 7F**).

[**0140**] The capacity of murine immunoregulatory DCs to induce immunotolerance to allogeneic $CD4^+$ T cells was inspected.

[**0141**] Allogeneic $CD4^+$ T cells (H-2^d) that had been stimulated with murine normal mature DCs (H-$_2{}^d$) in the primary culture strongly responded to secondary stimulation with murine normal mature DCs (H-2^d). Allogeneic $CD4^+$ T cells (H-$_2{}^b$) that had been stimulated with murine immunoregulatory DCs (H-2^d) exhibited low reactivity with the secondary stimulation with murine normal mature DCs (H-$_2{}^d$). Growth of $CD4^+$ T cells, however, was restored with the addition of IL-2 when secondary stimulation took place. In contrast, $CD4^+$ T cells (H-2^b) that had been stimulated with murine normal mature DCs (H-2^d) exhibited reactivity equivalent to the response of unprimed $CD4^+$ T cells (H-2^b) against secondary stimulation with mature normal DCs (H-2^q or H-2^k) derived from the unrelated mice. $CD4^+$ T cells that had been stimulated with murine immunoregulatory DCs (H-2^d) exhibited slightly weaker reactivity with mature normal DCs (H-2^q or H-2^k) derived from the unrelated mice (**FIG. 7O**). Similar results were attained with combinations of different mouse strains (**FIG. 7H**).

Example 9

Therapeutic Effects of Murine Immunoregulatory DCs on Acute GvHD after Allogeneic Transplantation

[**0142**] In order to analyze the therapeutic effects of murine immunoregulatory DCs on acute GvHD caused after allogeneic bone marrow transplantation, the influence of murine immunoregulatory DCs on lethal acute GvHD, which was developed in the recipient mice to which allogeneic bone marrow cells and spleen mononuclear cells had been transplanted, was inspected in the following manner. PBS (0.2 ml) consisting of bone marrow cells derived from allogeneic donor mice (1.5×10^7 cells/mouse) or PBS (0.4 ml) comprising the aforementioned bone marrow cells and spleen mononuclear cells (1.5×10^7 cells/mouse) were transplanted via injection into caudal veins of recipient mice (H-2^d or H-2^b, each group consisting of 5 individuals) irradiated with lethal doses of X-rays (10 Gy/mouse). Two or five days after transplantation, murine normal mature DCs derived from the syngeneic or allogeneic strains of the recipient mice, murine immunoregulatory DCs, or dihydroxyvitamin D_3-conditioned DCs were administered to the recipient mice in amounts of 1.5×10^4 to 5.0×10^6 cells/0.2 ml/mouse once or twice. The aforementioned recipient mice into which allogeneic bone marrow cells and spleen mononuclear cells had been transplanted were subjected to observation once a day until they died of GvHD or until 60 days had passed after the transplantation, in order to inspect their survival periods and changes in body weights. Allogeneic bone marrow cell- or spleen mononuclear cell (H-2^b)-transplanted recipient mice (H-2^d) developed significant symptoms of acute GvHD such as piloercetion, lowered motility, and decreased body weights within 6 days after transplantation, and all individuals died within 8 days after transplantation. Individuals in the group to which murine normal mature DCs (H-2^d) had been administered once in amounts of 1.5×10^6 cells/mouse 2 days after bone marrow transplantation died before acute GvHD was developed. However, recipient mice of the group to which murine immunoregulatory DCs (H-2^d) of their syngeneic mice had been administered once 2 days after bone marrow transplantation in amounts of 1.5×10^6 cells/mouse did not die, and survived until 60 days after transplantation. In this case, no or substantially no symptoms of acute GvHD were observed (**FIG. 8A**). Murine immunoregulatory DCs exhibited more potent therapeutic effects on acute GvHD than dihydroxyvitamin D_3-conditioned DCs (**FIG. 8B**). Similar results were attained with combinations of different mouse strains (**FIG. 8C**).

[**0143**] Single administration of a different number of murine immunoregulatory DCs, i.e., 1.5×10^4 cells/mouse,

1.5×10^5 cells/mouse, or 1.5 x 10^6 cells/mouse, was carried out 2 days after bone marrow transplantation, and the therapeutic effects on acute GvHD were found to vary in a dose-dependent manner (**FIG. 8D**). Also, when murine immunoregulatory DCs (1.5×10^4 cells/mouse or 1.5×10^5 cells/mouse) were administered 2 days after transplantation or 2 days and 5 days after transplantation and when murine immunoregulatory DCs (1.5×10^6 cells/mouse) were administered 5 days after transplantation, the survival of recipient mice was prolonged (**FIG. 8D**). In contrast, a single administration of murine immunoregulatory DCs (1.5×10^6 cells/mouse) 5 days after bone marrow transplantation significantly lowered the therapeutic effects. A single administration of murine immunoregulatory DCs (5.0×10^6 cells/mouse) allowed all recipient mice to survive (**FIG. 8D**).

Example 10

Influence of the Adminsitered Murine Immunoregulatory DCs on Immune Responses of Allogeneic Bone-Marroe-Transplanted Recipients and the Half-Lives Thereof

[**0144**] Donor-derived T-K^{b+} T cells in the spleen mononuclear cells of the recipient mice 5 days after bone marrow transplantation were analyzed. The contents of I-K^{b+} $CD3^+$ T cells, I-K^{b+} $CD4^+$ T cells, and I-K^{b+} $CD8^+$ T cells in the spleen mononuclear cells of the recipient mice to which murine normal mature DCs had been administered after bone marrow transplantation were significantly increased compared with those in the recipient mice to which DCs had not been administered. In contrast, I-K^{b+} $CD3^+$ T cells and I-K^{b+} $CD8^+$ T cells in the recipient mice to which murine immunoregulatory DCs had been administered significantly decreased compared with those in the recipient mice to which DCs had not been administered. Although there was no significant difference with regard to the I-K^{b+} $CD4^+$ T cell contents, they were significantly decreased (**FIG. 9A**).

[**0145**] Allogeneic reactivity of I-K^{b+} $CD4^+$ T cells against murine normal mature DCs in the recipient mice to which bone marrow had been transplanted was inspected. I-K^{b+} $CD4^+$ T cells prepared from recipient mice to which DC had not been administered or murine normal mature DCs had been administered strongly responded to murine normal mature DCs (H-2^d). In contrast, I-K^{b+} $CD4^+$ T cells prepared from recipient mice to which murine immunoregulatory DCs had been transplanted exhibited low reactivity with murine normal mature DCs (H-2^d), and this reactivity was restored with the addition of recombinant murine IL-2. The reactivity of those I-K^{b+} $CD4^+$ T cells to the mature normal DCs from unrelated mice (H-2^q) was lower than that of another recipients (**FIG. 9B**).

[**0146**] For the purpose of inspecting the cytotoxicity of donor-derived I-K^{b+} $CD8^+$ T cells against the recipients' tissues (H-2^d) in the bone marrow-transplant recipients, the cytotoxicity of I-K^{b+} $CD8^+$ T cells prepared from the recipients against P815 and EL4 was inspected. I-K^{b+} $CD8^+$ T cells derived from the recipients to which murine normal mature DCs had been administered exhibited higher cytotoxicity against P815 than that derived from the recipients to which DCs had not been administered. In contrast, the cytotoxicity against P815 of I-K^{b+} $CD8^+$ T cells derived from the recipients to which murine immunoregulatory DCs had been administered was significantly low. In these I-K^{b+} $CD8^+$ T cells, no or substantially no cytotoxicity against EL4 was observed. It was suggested that the cytotoxicity of I-K^{b+} $CD8^+$ T cells was specific to H-2^d (**FIG. 9C**).

[**0147**] The inflammatory cytokine content in the serum of the recipients 5 days after bone marrow transplantation was inspected. The content of IFN-γ, TNF-α, and IL-12 p40 in the serum of the recipients to which murine normal mature DCs had been administered was significantly higher than that in the recipients to which DCs had not been administered. In contrast, the content of IFN-γ, TNF-α, and IL-12 p40 in the serum of the recipients to which murine immunoregulatory DCs had been administered was significantly lower than that in the recipients to which DCs had not been administered (**FIG. 9D**).

[**0148**] For the purpose of inspecting the half-lives of murine immunoregulatory DCs that had been administered to the recipient mice, murine immunoregulatory DCs (H-2^d) to which carboxyfluorescein diacetate-succinimidyl estate (CFSE) had been added was administered to the bone marrow-transplanted recipients Migration thereof to the spleen was inspected using a flow cytometer. In the spleen mononuclear cells I day after administration of DCs, about 4% thereof were found to be $CFSE^+$ murine immunoregulatory DCs, and the half-life of the murine immunoregulatory DCs administered was approximately 18 days after administration (**FIG. 9E**). In order to inspect the stability of murine immunoregulatory DCs under inflammation-inducing conditions after transplantation in an organism, the expression of cell surface molecules and capacity for activating allogeneic T cells of murine immunoregulatory DCs prepared from the recipient mice (H-2^b) to which bone marrow (H-2^q) had been transplanted and murine immunoregulatory DCs (H-2^d) had been administered 5 days after transplantation were inspected (Table 1). This indicated that there were no or substantially no changes in properties of murine immunoregulatory DCs due to transplantation in an organism, and properties of murine immunoregulatory DCs were maintained even under inflammation-inducing conditions in an organism.

Example 11

Examination of the Involvement of Immunoregulatory T Cell Induction in Therapeutic Effects of the Administered Murine Immunoregulatory DCs in Allogeneic GvHD

[**0149**] Donor-derived $CD4^+$ T cells (H-2^b) were prepared from spleens of mice (H-2^d) after the transplantation of allogeneic bone marrow cells and spleen mononuclear cells (H-2^b) or spleens of mice (H-2^d) to which a variety of DCs (H-2^b) had been administered after the aforementioned transplantation 5 days after the transplantation. The ratios of CD25, CD152, and CD154 to be expressed were analyzed using FACS, and the results were compared with those of normal mice (H-2^b) without transplantation (**FIG. 10A**). Transplantation, administration of DCs, and preparation of donor-derived $CD4^+$ T cells were carried out in accordance with the method described in Example 6. In the group to which only allogeneic bone marrow cells and spleen mononuclear cells had been transplanted (recipients (H-2^d) of BMS (H-2^b)) and in the group to which murine normal mature DCs had been administered subsequent to the trans-

plantation (mDC (H-2^d)-treated recipients (H-2^d) of BMS (H-2^b)), the ratios of CD25 and CD154 to be expressed were higher than those in the case of normal mice. In contrast, the ratios of CD25 and CD152 to be expressed were higher in the group to which allogeneic bone marrow cells and spleen mononuclear cells had been transplanted and murine immunoregulatory DCs had been then administered (rDC (H-2^d)-treated recipients (H-2^d) of BMS (H-2^b))

[0150] As described above, donor-derived $CD4^+$ T cells (H-2^b) were prepared from spleens of mice (H-2^d) after the transplantation of allogeneic bone marrow cells and spleen mononuclear cells (H-2^b) or spleens of mice (H-2^d) to which a variety of DCs (H-2^b) had been administered after the aforementioned transplantation 5 days after the transplantation. Intracellular cytokines after the secondary stimulation carried out in the aforementioned manner were analyzed using FACS (**FIG. 10B**). Transplantation, administration of DCs, preparation of donor-derived $CD4^+$ T cells, and analysis of intracellular cytokines were carried out in the manner described above. In the group to which only allogeneic bone marrow cells and spleen mononuclear cells had been transplanted (recipients (H-2^d) of BMS (H-2^b)) and in the group to which murine normal mature DCs had been administered subsequent to the transplantation (mDC (H-2^d)-treated recipients (H-2^d) of DMS (H-2^b)), the IFN-γ-producing cell content and IL-2-producing cell content increased compared with those in normal mice (H-2^b). In contrast, the IL-10-producing cell content increased in the group to which allogeneic bone marrow cells and spleen mononuclear cells had been transplanted and murine immunoregulatory DCs had been then administered (rDC (H-2^d)-treated recipients (H-2^d) of BMS (H-2^b)).

[0151] Donor-derived $CD4^+$ $CD25^+$ T cells (H-2^b) from the spleen mononuclear cells of mice (H-2^d) to which a variety of DCs (H-2^d) had been administered after transplantation of allogeneic bone marrow cells and spleen mononuclear cells (H-2^b) were obtained, and CD152 and CD154 expression thereof was compared with those in $CD4^+$ $CD25^+$ T cells of normal mice (**FIG. 10C**). Transplantation and administration of DCs were carried out in the manner described above. $CD4^+$ $CD25^+$ T cells of normal mice were prepared from $CD4^+$ T cells of the spleen cells obtained in the manner described above using an anti-CD25 antibody (Clone PC61, BD PharMingen) and a magnetic-beads-coupled anti-rat IgG sheep antibody (Dynal). Donor-derived $CD4^+$ $CD25^+$ cells of mice that had undergone transplantation and administration of a variety of DCs were similarly prepared from the donor-derived $CD4^+$ T cells obtained in the manner described above using an anti-CD25 antibody and a magnetic-beads-coupled anti-rat IgG sheep antibody. Purity of the prepared $CD4^+$ $CD25^+$ cells was found to be 90% or higher as a result of analysis using FACS. CD154 and CD152 expression of the thus obtained cells were analyzed using FACS. In unprimed $CD4^+$ $CD25^+$ T cells obtained from normal mice (H-2^b), CD152 was constitutively expressed in some cells, although expression of CD154 was not observed as reported in the past (Takabashi et al., 2000, J. Exp. Med. 192, 303-309). In contrast, CD154 was expressed in most of donor-derived $CD4^+$ $CD25^+$ T cells of mice to which allogeneic bone marrow cells and spleen mononuclear cells had been transplanted and murine normal mature DCs had been then administered (mDC (H-2^d)-treated recipients (H-2^d) of BMS (H-2^b)). In most of the donor-derived $CD4^+$ $CD25^+$ T cells of mice to which allogeneic bone marrow cells and spleen mononuclear cells had been transplanted and murine immunoregulatory DCs had been then administered (rDC (H-2^d)-treated recipients (H-2^d) of BMS (H-2^b)), CD152 was expressed.

[0152] Donor-derived $CD4^+$ $CD25^+$ T cells (H-2^b) in the spleens were prepared from mice (H-2^d) to which allogeneic bone marrow cells and spleen mononuclear cells (H-2^b) had been transplanted and murine immunoregulatory DCs (H-2^d) had been then administered (2 days after transplantation), and changes in the ratios of CD152 to be expressed with the elapse of time were analyzed using FACS on 1, 3, 5, 10, 30, and 60 days after transplantation (**FIG. 10D**). Transplantation, administration of DCs, and preparation of donor-derived $CD4^+$ $CD25^+$ T cells were carried out in the manner described above. Compared with unprimed $CD4^+$ $CD25^+$ T cells of normal mice (H-2^b), the ratio of CD152 to be expressed was elevated after administration of murine immunoregulatory DCs and the high positive ratio was maintained until 60 days after administration in the $CD4^+$ $GD25^+$ T cells derived from mice to which allogeneic bone marrow cells and spleen mononuclear cells had been transplanted and murine immunoregulatory DCs had been then administered (rDC (H-2^d)-treated recipients (H-2^d) of BMS (H-2^b)).

[0153] Reactivity of $CD4^+$ T cells (H-2^b) stimulated with mature murine allogeneic DCs (mDCs H-2^d), that of unprimed $CD4^+$ $CD25^+$ T cells (H-2^b), and that of donor-derived $CD4^+$ $CD25^+$ $CD152^+$ T cells (H-2^b) of mice (H-2^d) to which allogeneic bone marrow cells and spleen mononuclear cells (H-2^b) had been transplanted and murine immunoregulatory DCs (H-2^d) had been transplanted were compared in the same manner as in Example 8 (**FIG. 10E**). The ratio of the number of T cells to that of mature murine allogeneic DCs was 10:1. Donor-derived $CD4^+$ $CD25^+$ T cells of mice to which allogeneic bone marrow cells and spleen mononuclear cells had been transplanted and immunoregulatory DCs had been then administered exhibited low response to mature murine allogeneic DC stimulation, as with the case of unprimed $CD4^+$ $CD25^+$ T cells. None of the cells responded to murine allogeneic immunoregulatory DCs stimulation (rDCs (H-2^d)). A variety of $CD4^+$ $CD25^+$ T cells (H-2b) were added to a mixed-culture system of $CD4^+$ T cells (H-2^b) at the time of the aforementioned mature murine allogeneic DC stimulation (H-2^d) in amounts consisting of the same number of cells as $CD4^+$ T cells so as to examine suppressing activity of $CD4^+$ $CD25^+$ T cells, and evaluation was carried out based on ^3H thymidine incorporation on the third day of culture. Donor-derived $CD4^+$ $CD25^+$ T cells of mice to which allogeneic bone marrow, cells and spleen mononuclear cells had been transplanted and immunoregulatory DCs had been then administered exhibited activity of suppressing the growth of $CD4^+$ T cells, as with the case of unprimed $CD4^+$ $CD25^+$ T cells, and this suppressing activity was more potent than that of unprimed $CD4^+$ $CD25^+$ T cells. When the haplotype (mDCs (H-2^q)) was different from that of murine immunoregulatory DCs (rDCs (H-2^d)) to which mature murine allogeneic DCs for stimulation had been administered, similar suppressing activity was observed. Thus, suppressing activity of $CD4^+$ $CD25^+$ T cells derived from mice to which allogeneic hone marrow cells and spleen mononuclear cells had been transplanted and murine immunoregulatory DCs had been then

administered was found to be antigen-nonspecific, as with the case of unprimed $CD4^+$ $CD25^+$ T cells.

[0154] In order to more precisely examine the level of suppressing activity of $CD4^+$ $CD25^+$ T cells shown in **FIG. 10E**, the number of $CD4^+$ $CD25^+$ T cells (H-2^b) to be added to the mixed-culture system of mature murine allogeneic DCs (H-2^b) with $CD4^+$ T cells (H-2^b) was varied (**FIG. 10F**). Transplantation and administration of DCs were carried out in the manner described above. Donor-derived $CD4^+$ $CD25^+$ T cells (H-2^b) of mice (H-2^d) to which allogeneic bone marrow cells and spleen mononuclear cells (H-2^b) had been transplanted and murine immunoregulatory DCs (H-2^d) had been then administered were prepared in the manner described above 5 days after the transplantation. A smaller number of donor-derived $CD4^+$ $CD25^+$ $CD152^+$ T cells of mice to which allogeneic bone marrow cells and spleen mononuclear cells bad been transplanted and immunoregulatory DCs had been then administered than unprimed $CD4^+$ $CD25^+$ T cells was sufficient to exhibit potent suppressing activity, and enhanced activity of immunoregulatory T cells was observed with the administration of murine immunoregulatory DCs.

[0155] The way that suppressing activity of donor-derived $CD4^+$ $CD25^+$ T cells was enhanced with the elapse of time, which was caused by administration of immunoregulatory DCs (H-2^d) after transplantation of allogeneic bone marrow cells and spleen mononuclear cells (H-2^b), was examined (**FIG. 10G**). Transplantation and administration of DCs were carried out in the manner described above, Donor-derived $CD4^+$ $CD25^+$ T cells (H-2^b) were prepared from mice (H-2^d) to which allogeneic bone marrow cells and spleen mononuclear cells (H-2^b) had been transplanted and murine immunoregulatory DCs (H-2^d) had been administered (2 days after transplantation) on 1, 3, 5, 10, 30, and 60 days after transplantation (rDC (H-2^d)-treated recipients (H-2^d) of BMS (H-2^b)) in the manner described above, and suppressing activity was examined in the same manner as with the case shown in **FIG. 10E**. The suppressing activity was equivalent to that of unprimed $CD4^+$ $CD25^+$ T cells on day 1, although suppressing activity was enhanced after the administration of immunoregulatory DCs, and high suppressing activity was maintained until 60 days after the transplantation.

[0156] Reactivity and suppressing activity of donor-derived $CD4^+$ $CD25^+$ T cells (H-2^b) of mice (H-2^d) to which allogeneic bone marrow cells and spleen mononuclear cells (H-2^b) had been transplanted and murine normal mature DCs (H-2^d) had been then administered were examined in the same manner as that shown in **FIG. 10E (FIG. 10H)**. Unlike the donor-derived $CD4^+$ $CD25^+$ $CD152^+$ T cells (H-2^b) or mice to which murine immunoregulatory DCs had been administered, the donor-derived $CD4^+$ $CD25^+$ $CD154^+$ T cells (H-2^b) of mice to which murine normal mature DCs had been administered exhibited more potent growth responses with mature murine allogeneic DCs stimulation (mDCs (H-2^d)) than the $CD4^+$ T cells (H-2^b). No activity of suppressing $CD4^+$ T cell growth when added to the mixed-culture system for mature murine allogeneic DCs and $CD4^+$ T cells was observed. Subsequently, the following experiment was carried out in order to examine the properties of suppressing activity observed in the donor-derived $CD4^+$ $CD25^+$ T cells (H-2b) of mice (H-2^d) to which allogeneic bone marrow cells and spleen mononuclear cells (H-2^b) had been transplanted and immunoregulatory DCs (H-2^d) had been then administered. At the outset, suppression assay similar to that shown in **FIG. 10E** was carried out in the presence of 100 U/ml of IL-2 to examine the influence of IL-2 on suppressing activity. In the presence of IL-2, suppressing activity of donor-derived $CD4^+$ $CD25^+$ T cells of mice to which allogeneic bone marrow cells and spleen mononuclear cells had been transplanted and murine immunoregulatory DCs had been then administered was partially attenuated. A transwell experiment was further carried out in order to examine the dependence of suppressing activity on cell contact in the following manner. Mature murine allogeneic DCs (H-2^d, 10^5 cells/well), $CD4^+$ T cells (H-2^b, 10^6 cells/well), and donor-derived $CD4^+$ $CD25^+$ T cells (H-2b, 10^6 cells/well) of mice to which allogeneic bone marrow cells and spleen mononuclear cells had been transplanted and murine immunoregulatory DCs had been then administered were mixed in a 24-well plate (coculture). Alternatively, $CD4^+$ T cells and mature murine allogeneic DCs were cultured separately from $CD4^+$ $CD25^+$ T cells and mature murine allogeneic DCs using a transwell for 4 days (separated culture). Normal mature murine DCs were then removed, T cells remaining thereafter were transferred to a 96-well plate in amounts of 10^5 cells/well, and ^3H thymidine incorporation 5 days after the initiation of culture was evaluated. When cell contact between $CD4^+$ T cells and $CD4^+$ $CD25^+$ T cells was blocked with a transwell, suppressing activity disappeared. This demonstrated the dependence of suppressing activity on cell contact.

[0157] Roles of IL-10-producing $CD4^+$ T cells and those of $CD4^+$ $CD25^+$ T cells in therapeutic and ameliorating effects of murine immunoregulatory DCs on acute GvHD developed after allogeneic bone marrow transplantation were inspected in the following manner (**FIG. 10I**). In accordance with Example 9, allogeneic bone marrow cells and spleen mononuclear cells (H-2^b) were transplanted to recipient mice (H-2^d), and murine immunoregulatory DCs (1.5×10^6 cells/mouse) were administered thereto on the second day. Thereafter, an anti-CD25 antibody (Clone PC61, BD PharMingen), an anti-IL-10-neutralizing polyclonal antibody (model AB-417-NA, R&D Systems, Minneapolis, Minn.), an anti-TGF-β-neutralizing antibody (Clone 1D11, R&D Systems, Minneapolis, Minn.), or control rat IgG were administered intravenously to mice in amounts of 500 μg/mouse 3, 5, 7, 9, 10, 13, and 15 days after transplantation. Administration of the aforementioned anti-CD25 antibody resulted in disappearance of 98% or more of $CD4^+$ $CD25^+$ T cells in the spleens of the recipient mice to which murine immunoregulatory DCs had been administered 16 days after transplantation. As a result, the effects of murine immunoregulatory DCs for ameliorating acute GvHD were significantly deteriorated by the administration of an anti-CD25 antibody or anti-IL-10 antibody, although no influence was imposed upon the aforementioned effects by the administration of an anti-TGF-β antibody or control rat IgG. Simultaneous administration of an anti-CD25 antibody and an IL-10 antibody more potently suppressed the effects of murine immunoregulatory DCs to ameliorate acute GvHD (**FIG. 10I**). Effects of single administration of $CD4^+$ $CD25^+$ immunoregulatory T cells, $CD4^+$ $CD25^+$ $CD154^+$ T cells, or $CD4^+$ $CD25^+$ $CD152^+$ T cells to recipient mice to which allogeneic bone marrow cells had been transplanted were inspected. Allogeneic bone marrow cells and spleen mononuclear cells (H-2^b) were transplanted to recipient mice

($H\text{-}2^d$) in accordance with Example 9, and the unprimed $CD4^+$ $CD25^+$ immunoregulatory T cells, $CD4^+$ $CD25^+$ $CD154^+$ T cells, and $CD4^+$ $CD25^+$ $CD152^+$ T cells prepared on the second day ($H\text{-}2^b$) were administered intravenously thereto once. As a result, the $CD4^+$ $CD25^+$ $CD152^+$ T cells exhibited more potent suppressing effects on acute GvHD compared with the unprimed $CD4^+$ $CD25^+$ immunoregulatory T cells In contrast, $CD4^+$ $CD25^+$ $CD154^+$ T cells were apt to significantly exacerbate the suppressing effects (**FIG. 10J**).

Example 12

Analysis of Phenotype of Cells Induced from Allogeneic $CD4^+$ $CD25^-$ T Cell in vitro Using Murine Immunoregulatory DCs

[**0158**] $CD4^+$ mononuclear cells derived from spleens of C57BL/6 mice ($H\text{-}2^b$) were prepared in the manner described above, and $CD25^+$ cells were removed therefrom using a rat anti-CD25 antibody and a magnetic-beads-coupled goat anti-rat IgG antibody. Thus, $CD4^+$ CD25T cells with purity of 95% or higher wcrc prepared. The resulting T cells were stimulated by being subjected to mixed culture with murine normal mature DCs or murine immunoregulatory DCs prepared from BALB/c mice ($H\text{-}2^d$) in a manner described above at a mixing ratio of 10:1 (T cells:DCs). Five days after the initiation of mixed culture, DC fractions were removed using a mouse anti-$I\text{-}K^d$ antibody and a magnetic-beads-coupled goat anti-mouse IgG antibody to prepare T cell fractions, and the resultants were analyzed by flow cytometry. As shown in **FIG. 11**, the $CD154^+$ cell content was high and the $CD152^+$ cell content was low in T cells stimulated with murine normal mature DCs. In contrast, the $CD154^+$ cell content was significantly lower and the $CD152^+$ cell content was significantly higher in T cells stimulated with murine immunoregulatory DCs than in 1 cells stimulated with murine normal mature DCs. This indicates that murine immunoregulatory DCs can also induce $CD152^+$ cells in vitro.

Example 13

Influence of Murine Immunoregulatory DCs on Graft-Versus-Leukemia Effects in Tumor-Bearing Mice Transplanted with Bone Marrow Cells and Spleen Mononuclear Cells

[**0159**] P815 mastocytomas (2×10^5 cells/0.2 ml, $H\text{-}2^d$, RIKEN Cell Bank, Tsukuba, Japan) were administered intravenously to BALB/c mice ($H\text{-}2^d$, each group consisting of 5 individuals). Two days thereafter, mice were systemically irradiated with lethal doses of radiation (10 Gy/mouse, source: ^{60}Co, MBR-1505R2, Hitachi Medical, Tokyo, Japan), and a group to which host-incompatible bone marrow nucleated cells (BM, 1.5×10^7 cells suspended in 0.2 ml of phosphate buffered saline) prepared in a manner described above or host-incompatible bone marrow nucleated cells and spleen mononuclear cells (BMS, a mixture of 1.5×10^7 cells each suspended in 0.4 ml of phosphate buffered saline) were administered via tail veins were prepared. The influence of murine immunoregulatory DCs (rDCs) on graft-versus-leukemia effects was observed by administering murine immunoregulatory DCs to the group to which host-incompatible bone marrow nucleated cells and spleen mononuclear cells (BMS) (a mixture of 1.5×10^7 cells each suspended in 0.4 ml of phosphate buffered saline) had been administered on the second day.

[**0160**] **FIG. 12** shows the results of observation concerning the survival of mice (**FIG. 12A**), changes in body weights (**FIG. 12B**), and weights of livers or spleens (**FIG. 12C**). All mice that bad been systemically irradiated with radiations died, their body weights decreased, and splenohepatomegaly was observed until the 12th day. In contrast, life prolongation was observed until the 30th day in the group to which only BM had been administered, although death involving splenohepatomegaly, which was presumably caused by leukemia, was observed. While all mice died in the period up until the 8th day in the group to which BMS had been administered, life prolongation of 60 days or longer and liver and spleen weight increases were observed in the group to which murine immunoregulatory DCs had been further administered. This indicates that anti-graft-versus-host disease effects were observed and graft-versus-leukemia effects could be maintained in the group to which murine immunoregulatory DCs had been administered.

Example 14

Murine Immunoregulatory DCs Suppress Developed Type II Collagen-Induced Arthritis

[**0161**] Activity of murine immunoregulatory DCs upon type II collagen-induced arthritis was examined. Arthritis was induced by subcutaneously administering 100 μg of bovine type II collagen (CII) to DBA/1 mice. CII, which had been used for sensitization, was administered as an emulsion together with Freund's complete adjuvant (Difco, Detroit, Mich.). Arthritis was observed every other day, and the results of observation were scored. Criteria were as follows: 0=no change; 1=slight erythema and edema; 2=advanced erythema and edema; and 3=deformity involving joint flexion. The maximal score would be 12 for total of 4 criteria. The day when arthritis had been developed was determined to be day 1, and murine normal mature DCs or murine immunoregulatory DCs were administered through caudal veins on that day. A group to which DCs had not been administered was provided as a control. Murine normal mature DCs were prepared in a manner as described in Example 6, these cells were cultured in the presence of CII (1 μg/ml) for 24 hours, and the culture products were then administered, As a result, murine immunoregulatory DCs more significantly suppressed the development of arthritis compared with the control group and the group to which murine normal mature DCs had been administered (**FIG. 13A**). Ten days after the development of arthritis, T cells derived from murine inguinal lymph nodes and subgenual lymph nodes were isolated, and their reactivity with murine normal mature DCs cocultured with CII was examined in the following manner. Lymphocytes were isolated from murine inguinal lymph nodes and subgenual lymph nodes using a

Lympholyte-M (Cedarlane), and the isolated lymphocytes were subjected to negative selection using anti-Ly76, B220, Ly-6G, I-A/I-E, and a magnetic-beads-coupled anti-rat IgG antibody to prepare T cells. CII-pulsed DCs were prepared in the following manner. Murine DCs were cultured in the presence of CII (1 μg/ml) for 24 hours, and the obtained DCs were further cultured in the presence of LPS (1 μg/ml) for 3 days. The obtained T cells (10^5 cells) and X-ray (15 Gy)-irradiated murine normal mature DCs (10^3 to 5×10^4 cells) were cultured on a 96-well plate for 5 days, and cell growth assay was carried out. As a result, T cells derived from mice to which murine normal mature DCs had been administered exhibited significantly elevated reactivity compared with the control group, and the reactivity of T cells derived from mice to which murine immunoregulatory DCs had been administered was lowered **(FIG. 13B)**

Example 15

Effects of Immunoregulatory DCs for Suppressing Experimental Autoimmune Encephalomyelitis (EAE)

[0162] Partial peptides of myelin oligodendrocyte glycoproteins (200 μg, MOG35-55: MEVGWYRSPFSRVVHLYRNGK: SEQ ID NO: 1, Qiagen) and heat-killed *Mycobacterium tuberculosis* H37Ra (600 μg, Difco) were added to complete Freund's adjuvant (CFA, Difco) to prepare an emulsion. The thus-prepared emulsion was administered intradermally to the backs of 8-week-old C57BL/6 mice for immunization on day 0. One day thereafter, pertussis toxin (Seikagaku Corporation) was administered intraperitoneally to the mice in amounts of 400 ng/mouse to induce experimental autoimmune encephalomyelitis (EAE). EAE is an experimental animal model of multiple sclerosis. C57BL/6 mouse-derived immunoregulatory DCs (2×10^5 cells) were administered intravenously to the mice on the second day, and a phosphate buffered saline (PBS) solution was administered to the control group. In this experiment, groups of mice each consisting of 10 individuals were employed. Immunoregulatory DCs were induced from the murine bone marrow cells by the process described in Example 6. In this experiment, 26 μg/ml of MOG35-55 was added to the medium during the final 2 days of culturing at the time of LPS stimulation for inducing immunoregulatory DCs. The severity of EAE symptoms was scored as follows: 0=normal; 1=loss of tail tonicity; 2=impairment in righting reflex; 3=partial paralysis of hind legs; 4=complete paralysis of hind legs; 5=paralysis of fore and hind legs; and 6=death. Concerning the EAE scores, the area under curve (AUC) was determined for each individual for the purpose of statistical tests.

[0163] **FIG. 14** shows changes in the mean EAE scores. In the control group, the development of EAE was first observed about 1 week after the induction of EAE, the rate of development reached its peak about 2 weeks thereafter, and this status was maintained until the fourth week. In contrast, the development of EAE was first observed I week after the induction of EAE in only a few individuals in the group to which immunoregulatory DCs had been administered, although the symptoms thereof were milder than those of the control groups. Table 2 shows values for a variety of parameters associated with the EAE symptoms obtained in an experiment identical to that of **FIG. 14**. In the group to which immunoregulatory DCs had been administered, the EAE scores (AUC), the maximal scores, and the rate of development were significantly lowered compared with those of the control group. This indicates that the administration of immunoregulatory DCs can suppress multiple sclerosis.

TABLE 2

	Effect of immuno reguratory DC adiminsitration on parameters of EAE (Mean ± SD)				
Group	n	EAE score (AUC)	Day of onset	Maximum score	Onset (%)
Control group	10	37.3 ± 4.9	9.5 ± 1.9	2.1 ± 0.3	100
DCreg adiministered group	10	7.8 ± 12.2**	12.5 ± 5.2	0.7 ± 0.9**	40#

#p < 0.05 (χ-squared test),
**p < 0.01 (t-test)

Example 16

Effects of Immunoregulatory DCs for Suppressing Delayed-Type Hypersensitivity (DTH)

[0164] Chicken ovalbumin (OVA, 100 μg) was added to complete Freund's adjuvant (CFA, Sigma) to prepare an emulsion. The thus-prepared emulsion was administered intradermally to the backs of 9-week-old C57BL/6 mice for immunization and sensitization on day 0. Ten days thereafter, 20 μl of a PBS solution containing 10 μg of OVA was administered to the auricle to induce delayed-type hypersensitivity (DTH) On the following day, a piece of the auricle (diameter: 5 mm) surrounding the site to which OVA had been administered was removed using a skin biopsy puncher, it was weighed using an electronic balance, and the result was adopted as an indicator for the severity of DTH. C57BL/6 mouse-derived immunoregulatory DCs (1.5×10^6 cells) were administered intravenously to the mice on the first day, and a phosphate buffered saline solution was administered to the control group. In this experiment, a group that was not sensitized with OVA was provided in addition to the control group. The group that was not sensitized with OVA consisted of 5 mice, and other groups each consisted of 10 mice. Immunoregulatory DCs were

induced from the murine bone marrow cells by the process described in Example 6. In this experiment, 2 mg/ml of OVA was added to the medium during the final 2 days of culturing at the time of LPS stimulation for inducing immunoregulatory DCs.

[0165] **FIG. 15** shows the effects of DTH suppression. In the group to which immunoregulatory DCs had been administered, DTH was significantly suppressed than in the control group. DTH is a typical Th1 response. This indicates that the administration of immunoregulatory DCs can suppress autoimmune diseases or hyperinflammatory responses caused by Th1 responses.

[0166] Industrial Applicability

[0167] As described in the Examples, immunoregulatory DCs stimulated with IL-10 and TGF-β induce antigen-specific anergy to T cells and suppress reactivation of activated T cells (Example 2). Also, the aforementioned immunoregulatory DCs suppress graft-versus-host disease after xenogeneic transplantation caused by T cells (e.g., Example 5). Further, immunoregulatory DCs suppress immune-related diseases (Example 14). As described in these Examples, the immunoregulatory DCs of the present invention suppress rejection caused along with cell, organ, or tissue transplantation, have therapeutic effects on graft-versus-host disease while maintaining graft-versus-leukemia effects, and also have therapeutic effects on autoimmune and allergic diseases.

[0168] All publications cited herein are incorporated herein in their entirety. A person skilled in the art would easily understand that various modifications and changes of the present invention are feasible within the technical idea and the scope of the invention as disclosed in the attached claims. The present invention is intended to include such modifications and changes.

SEQUENCE LISTING

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1 5 10 15

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20

1. A method of preparing human immunoregulatory dendritic cells by culturing human dendritic cells or their precursor cells in vitro with IL-10 and TGF-β.

2. The method of preparing human immunoregulatory dendritic cells according to claim 1, wherein the human dendritic cells are derived from human monocytes.

3. A method of preparing human immunoregulatory dendritic cells comprising culturing human monocytes in the presence of GM-CSF, IL-4, IL-10, and TGF-β.

4. The method of preparing human immunoregulatory dendritic cells according to claim 3, wherein culture is further conducted in the presence of at least one of TNF-α and LPS.

5. The method of preparing human immunoregulatory dendritic cells according to any one of claims 1 to 4, wherein culture is further conducted in the presence of an antigen existing in a tissue or organ associated with a disease to be treated.

6. The method of preparing human immunoregulatory dendritic cells according to claim 5, wherein the disease is an autoimmune or allergic disease.

7. The method of preparing human immunoregulatory dendritic cells according to claim 5, wherein the disease is rheumatoid arthritis or multiple sclerosis.

8. Human immunoregulatory dendritic cells prepared by the method according to any one of claims 1 to 7.

9. The human immunoregulatory dendritic cells according to claim 8, wherein expression levels of CD83, CD40, CD80, and CD86 are significantly lower than those in mature human dendritic cells that were not cultured in the presence of both IL-10 and TGF-β.

10. The human immunoregulatory dendritic cells according to claim 8 or **9**, which are capable of inducing antigen-specific anergy to allogeneic $CD4^+$ T cells in vitro, suppressing reactivation of activated allogeneic $CD4^+$ T cells and $CD8^+$ T cells, inducing allogeneic and naive $CD4^+$ T cells and $CD8^+$ T cells to become $CD4^+$ $CD25^+$ immunoregulatory T cells and $CD8^+$ $CD28^-$ immunoregulatory T cells, respectively, and inducing immune-suppressing responses such as the suppression of graft-versus-host disease after xenogeneic transplantation in a human T cell-

transplanted immunodeficient mouse through administration of the aforementioned cells to which xenoantigens have been imparted.

11. A pharmaceutical composition comprising the human immunoregulatory dendritic cells according to any one of claims 8 to 10.

12. The pharmaceutical composition according to claim 11, which suppresses graft rejection caused along with cell, organ, or tissue transplantation.

13. The pharmaceutical composition according to claim 11, which can be used for treating graft-versus-host disease.

14. The pharmaceutical composition according to claim 11, which can be used for treating an autoimmune or allergic disease.

15. The pharmaceutical composition according to claim 11, which can be used for treating rheumatoid arthritis or multiple sclerosis.

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